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(54) Title: SINGLE PRIMER AMPLIFICATION OF POLYNUCLEOTIDE HAIRPINS

(57) Abstract

Disclosed is a method of exponentially amplifying a polynucleotide hairpin using a single primer. The method can be used to amplify double-stranded polynucleotides and to detect hairpin, double-stranded and single-stranded polynucleotides.

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SINGLE PRIMER AMPLIFICATION OF POLYNUCLEOTIDE HAIRPINS

Background

Polymerase chain reaction (PCR) is currently used to amplify minute quantities of polynucleotide. However, PCR requires two oligonucleotide primers which hybridize to the polynucleotide being amplified. Consequently, PCR can only be used if the polynucleotide sequence within two separated regions of the polynucleotide being amplified are known. Therefore, PCR cannot be used to amplify regions of unknown sequence which flank a region of known sequence. Inverse PCR is currently used to amplify flanking regions (see Ochman et al,. Genetic applications of an inverse polymer chain reaction. *Genetics*, 120:621-625 (1988)). However, inverse PCR requires an inefficient circularization step which requires a dilute solution of DNA. Consequently, there is currently a need for new methods of polynucleotide amplification which can employ a single primer and thereby be used for applications such as the amplification of flanking regions.

Summary of the Invention

It has been unexpectedly found that a polynucleotide hairpin can be exponentially amplified using a single primer and a template dependent polynucleotide polymerase. This discovery has been made, despite prior teachings in the art that single primer polymerase chain reaction (PCR) of DNA hairpins was unsuccessful under various conditions (Black et al., *Nucleic Acids Research* 22:1315 (1994)). Methods of amplifying polynucleotide hairpins, (e.g. hairpin DNA), double stranded polynucleotides (e.g. double stranded DNA), single stranded polynucleotides (e.g. single stranded DNA and RNA), and cDNA with a single primer are

disclosed, as are methods of detecting hairpin single and double stranded polynucleotides.

One embodiment of the present invention is a method of copying a hairpin polynucleotide, e.g. hairpin DNA. The method comprises providing at least one hairpin polynucleotide. The hairpin polynucleotide is combined with a template dependent polynucleotide polymerase, nucleoside triphosphates and an excess of a single polynucleotide primer. The single polynucleotide primer is capable of hybridizing to a site on the 3' portion of the hairpin polynucleotide suitable for primer extension such that an extension product synthesized from the single primer can serve as a template for the synthesis of an extension product of the single primer. The primer site is situated such that the primer is extended into the stem or duplex portion of the hairpin. The base-pairing of the hairpin polynucleotide is then disrupted, thereby dissociating the two complementary segments and converting the polynucleotide into a non-duplex or single stranded form. This product is then subjected to hybridization or annealing conditions such that at least the 3' portion of the primer can hybridize to the primer binding site on the hairpin polynucleotide. This product is exposed to conditions suitable for primer extension, thereby producing the extension product, which is a complementary copy of a portion of the hairpin polynucleotide. The extension product is believed to be in the form of a hairpin. The disruption, annealing, and primer extension steps can be repeated, thereby copying and producing an exponential amplification of the hairpin polynucleotide and the extension product.

The method of the present invention has many advantages. Because only a single primer is required, less knowledge of the sequence of the target DNA is required than with traditional polymerase chain reaction methods,

which require at least two primers. This method is useful for the amplification and sequencing of unknown regions of DNA flanking regions of known sequence. Applications of the present method include gene or chromosome walking, amplification and sequencing of cDNA, and detection of the presence of minute amounts of a specific DNA in a sample.

Description of the Figures

Figure 1A-1G shows a hairpin polynucleotide with a double stranded portion and a cap portion and the expected products of single primer amplification of a hairpin polynucleotide. It also shows a single stranded closed circular polynucleotide which is equivalent to two hairpins end to end, and the expected amplification products.

Figure 2A-2K is a diagram showing the steps in one cycle of single primer exponential amplification of a hairpin polynucleotide.

Figure 3 is a diagram of a hairpin adapter showing the cap or loop region, the stem region, and the ligatable end region.

Figure 4A-4B shows a hairpin adapter (SEQ ID NO: 1) with a 4 base 5' overhang and a hairpin adapter (SEQ ID NO: 2) with a 2 base 3' overhang.

Figure 5A-5F is a diagram showing the use of a DNA hairpin adapter which has a phosphorylated 5' end and a site complementary to a sequencing primer and which is ligatable to a double stranded DNA. Figure 5 also shows the use of this adapter for amplifying and sequencing double stranded DNA to which it can be ligated.

Figure 6A-6C is a diagram showing formation of a hairpin DNA from single stranded DNA.

Figure 7A-7B is a diagram showing a single stranded DNA with a 3' shepherd's crook and the conversion of this DNA into hairpin DNA suitable for single primer hairpin amplification.

Figure 8A-8F is a diagram showing a single stranded DNA with a 5' shepherd's crook and the conversion of this DNA into hairpin DNA suitable for single primer hairpin amplification.

Figure 9A-9G is a diagram showing the preparation and conversion of mRNA into hairpin DNA suitable for single primer hairpin amplification.

Figure 10A-10G is a diagram showing the production of hairpin DNA from single stranded RNA or DNA.

Figure 11 shows the sequences of adapters C77 (SEQ ID NO: 3), C78 (SEQ ID NO: 4) and D15 (SEQ ID NO: 5) and of primers B96 (SEQ ID NO: 6), D13 (SEQ ID NO: 7), C72 (SEQ ID NO: 8) and B36 (SEQ ID NO: 9).

Figure 12 is a diagram showing an *E. coli* 16S rDNA gene with restriction sites and an amplification primer binding site.

Figure 13A-13B shows hairpin adapter C78 (double lines) ligated at EcoR I site to *E. coli* DNA, (single lines) and the positions of primers C72 and B96. Also shown is the expected product obtained by amplifying this target DNA with single primer C72.

Figure 14A-14B shows adapter C77 (double lines) ligated at EcoR I site to *E. coli* DNA, (single lines) and the positions of primers C72, and B36. Also shown is a portion of the expected product SEQ ID NO: 10 obtained by amplifying this target DNA with single primer C72.

Figure 15 shows adapter C78 (double lines) ligated at *EcoR I* site to *E. coli* DNA (single lines) and the positions of primers C72 and B36.

Figure 16A-16B shows adapter D15 (double lines) ligated at the *Age I* site to *E. coli* DNA, (single lines) and the positions of primers C72, B36 and D13. Also shown is the expected product SEQ ID NO: 11 obtained by amplifying this target DNA with single primer C72.

Figure 17A-17D is a diagram showing a single stranded polynucleotide having regions D, C, B and A in the 5′ to 3′ direction, a probe having a 3′ region complementary to region A, and a 5′ region identical to B. The diagram also shows the preparation of a single stranded DNA capable of forming a 5′ shepherd's crook from the probe using the single stranded polynucleotide as a template.

Detailed Description of the Invention

The present invention is a method of exponential amplification of a hairpin polynucleotide with a single primer to produce two complementary products as described below. A polynucleotide can be DNA, RNA or analogues thereof. Examples include peptide nucleic acids (PNA), phosphothiolate nucleotides or other analogues which give specific hybridization to polynucleotides and which can be extended by a template dependent polynucleotide polymerase in the presence of nucleoside triphosphates.

A "hairpin polynucleotide", as used herein, is a single stranded polynucleotide having two regions which are sufficiently complementary that they hybridize to each other. Preferably, the two regions are completely complementary. As shown in Figure 1A, a hairpin polynucleotide is composed of two portions: a "stem" and a "cap" or "loop" region. In the "stem" region, complementary bases are paired in a typical antiparallel duplex. If the two complementary portions of the polynucleotide are separated by bases which do not form a complementary structure, then the bases form a single stranded loop off the end of the stem. A single stranded polynucleotide in which the two complementary regions comprise the entire molecule is said to be a "perfect hairpin". A perfect hairpin has a "cap" portion that is generally about four bases in length. As used herein, the term "loop" also encompasses a cap unless specified

otherwise. A loop can contain from 3 to about three thousand bases. Also included in the definition of hairpin polynucleotide is a circular polynucleotide having two regions that are sufficiently complementary that they hybridize to each other. In this case there is a loop portion at both ends of the double stranded stem portion as shown in Figure 1E.

In the present invention, the nuclectides in the double stranded portion of the hairpin generally outnumber the nucleotides in the loop portion. For example, the double stranded portion of the hairpin can comprise about 60% or more of the nucleotides. In some applications, the double stranded portion of the hairpin can comprise more than about 90% of the nucleotides and, at times, more than about 99% of the nucleotides.

A hairpin polynucleotide that is not circular has a 3' end and a 5' end. The portion in the stem of the hairpin with the 3' end is referred to as the "3' portion". The portion in the stem of the hairpin with the 5' end is referred to as the "5' portion".

Each reaction product contains a copy of at least a portion of the stem of the double stranded portion of the hairpin. These two reaction products can be produced, provided that 1) the sequence at a site (referred to as the "primer binding site") within the 3' portion of the hairpin polynucleotide is known in sufficient detail that oligonucleotides, referred to as "primers", can be synthesized which will hybridize to the site and 2) that a small amount of the hairpin polynucleotide is available to initiate the reaction.

A "primer" is a polynuclectide containing a sequence at its 3'-end hybridizable with a primer binding site. The primer may be longer than the primer binding site if the extension is at the 5' end. 5' extensions are often used to introduce novel sequences at the end of an amplified

product for cloning or other purposes. Usually, the number of bases in a primer will be at least as great as the primer binding site and generally from 8 to about 2,500 and preferably 15 to 100 nucleotides. The primer is an oligonucleotide, which, after hybridization to the target, acts as a point of initiation of synthesis when placed under appropriate conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced. Appropriate conditions occur, for example, in the presence of nucleoside triphosphates, and an inducing agent such as a template dependent polynucleotide polymerase and at a suitable temperature, pH, ionic strength and Mg^{++} concentration (or other suitable divalent cation required according to the particular polymerase used). A primer represents a 5' terminus of the resulting extension product.

The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. A primer can be obtained from a source in which it occurs naturally (e.g., by a purified restriction digest) or can be synthesized.

In the method of the present invention, the primer binds to the primer binding site, which is located on the 3' portion of the hairpin polynucleotide.
The primer binding site is separated from the loop by a duplex region referred to as the "proximal stem". The region of the stem extending beyond the primer binding site is referred to herein as the "distal stem".
The proximal stem is at least 8 base pairs in length, and is typically at least about 100 base pairs. In some applications the proximal stem may be as large as 2 kilobase pairs, and at times more than 40 kilobase pairs in length. As a result, primer extension by a template

dependent polynucleotide polymerase copies into a base paired region of the stem before reaching the loop or cap region, i.e. the polymerase first copies proximal stem, before reaching the loop. Primer extension may be more efficient for polymerases which possesses the property of strand displacement without the aid of helicases (see A. Kornberg and T. A. Bader, DNA replication, second ed. W. H. Freeman and Co., New York (1992)).

Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10^6:1 primer:template) of the single oligonucleotide primer is used in hairpin amplification. When the method described here is being used for diagnostic applications, the amount of template, i.e. the amount of hairpin in polynucleotide to be copied, may not be known, so that the amount of primer relative to the amount of template polynucleotide cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of target polynucleotide when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred for a high degree of amplification.

Suitable template dependent polynucleotide polymerases include DNA polymerases and RNA polymerases and reverse transcriptases. DNA polymerases which are not stable at high temperatures are suitable and include DNA polymerase I Klenow Fragment, T4 DNA polymerase, T7 DNA polymerase, and others. However, thermally stable DNA polymerases are preferred when more than one cycle of hairpin amplification is to be performed and include DNA polymerases from *Thermus aquaticus*, *Thermus thermophilus*, *Thermus* sp., *Thermococcus litoralis*, *Pyrococcus furiosus*, *Pyrococcus woesei*, *Pyrococcus* sp. Additional thermostable DNA polymerases are known (A.K. Bej and M.H. Mahbubani, "Thermostable DNA

polymerases for *in vitro* DNA amplifications," in H.G. Griffin and A.M. Griffin eds. PCR Technology: Current Innovations, CRC Press, Ann Arbor (1994)). Polymerases which naturally lack or have been modified to lack 5'-3' exonuclease activity and which have strand displacement activity, such as *Thermoccus litoralis* are preferred. RNA polymerases include *E. coli* RNA polymerase, SP6, T3 and T7 RNA polymerases, and others as well as those from thermophilic organisms. Reverse transcriptases from Avian Myeloblastosis Virus, Moloney Murine leukemia Virus, and other viral and bacterial sources, including those from thermophilic viruses or bacteria may be used.

Disrupting the base-pairing of the hairpin polynucleotide refers to dissociating the complementary portions of the hairpin stem as shown in Figure 2, by subjecting the hairpin polynucleotide to denaturing conditions. The double stranded portion of the hairpin polynucleotide is thereby dissociated, partially separating the two complementary portions of the hairpin This strand separation can be accomplished by any suitable method including chemical or enzymatic means. A preferred method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation may involve temperatures ranging from about 80°C to 105°C for times ranging from about 0.5 to 10 minutes, preferably at about 94°C for about 0.5 to 1 minute. Strand separation may also be induced by an enzyme from the class of enzymes known as DNA helicases which separate strands of duplex DNA and are described in A. Kornberg and T. A. Baker in "DNA replication", 2nd ed., pp 355-378 (Freeman and Co., New York, (1992). The reaction conditions suitable for separating the strands of nucleic acids with helicases are described in Cold Spring Harbor Symposia on Quantitative Biology, Volume XLII, "DNA Replication and Recombination"

(New York: Cold Spring Harbor Laboratory, (1978)), B. Kuhn *et al.*, and "DNA Helicases", pp. 63-67.

The denatured hairpin polynucleotide and primer are then exposed to conditions suitable for hybridization or annealing of the primer to the primer binding site on the hairpin polynucleotide. Under these hybridization conditions, the hairpin polynucleotide returns to its hairpin conformation with the bound primer as illustrated in Figure 2C and D. Because of the molar excess of primer and the greater mobility of the primer relative to the hairpin polynucleotide, the primer can usually be expected to bind before the hairpin structure is completely reformed.

The hairpin polynucleotide is then exposed to conditions suitable for primer extension. The primer is extended by the template dependent polynucleotide polymerase in the direction of the loop. The product obtained has the molecular weight of a single stranded polynucleotide, consistent with the product forming a hairpin structure, rather than a double stranded polynucleotide typically formed in traditional PCR amplification. Consequently, extension products of the present invention can be distinguished from PCR or other single primer amplification products on the basis of molecular weight; the hairpin product is one half the molecular weight of the double stranded product obtained from traditional amplification procedures (see Examples 1-4). Although Applicant does not wish to be bound by a particular mechanism, this result is consistent with a strand rearrangement occurring during the course of primer extension. An example of strand rearrangement is shown in Figure 2.

If the hairpin polynucleotide is a perfect hairpin (all bases complementary) then there is only one amplification product. However if the hairpin

polynucleotide has a loop, there are two complementary amplification products as shown in Figure 1. The loop portion of the first extension product, X', is complementary to the loop portion of the starting material, X, as shown in Figure 1C. The stem of the extension product contains the primer as its 5' end. The complementary 3' strand contains a copy of the primer binding site. Therefore, additional primer can hybridize to the first hairpin extension product and to the hairpin starting material following subsequent steps of denaturation and annealing. The first extension product can therefore serve as a template for primer extension. The loop of this second primer extension product, X, is complementary to the loop of the first primer extension product and is identical to the loop of the starting hairpin polynucleotide, as shown in Figure 1D. The stem portion of the products between the primer binding site and the loop, and the complement to the primer binding site and the loop are identical to the starting material.

Additional cycles of denaturation, annealing, and extension will produce exponential amplification of the products shown in Figure 1, panels C & D. These copies are referred to herein as "amplified polynucleotide or "amplicon". The cycles can be repeated as often as required to produce the desired level of exponential amplification. This process is referred to as "hairpin amplification". If the initial polynucleotide is actually a closed circle as in Figure 1E, then amplified products shown in Figure 1F are expected from amplification with primer 1 and products shown in Figure 1G, are expected from amplification with primer 2.

Conditions suitable for denaturation, annealing, and primer extension are given below. It is to be understood that many variations to these conditions are known in the art or can be made which can be applied to the single

primer amplification method described herein. Such variations and modifications are within the scope of the present invention and are discussed in: A. Erlich ed. "PCR technology: principles and applications for DNA amplification", M. Stockton Press, New York (1989); M.A. Innis et al., eds, "PCR Protocols: A Guide to Methods and Applications", Academic Press, San Diego (1990); H.G. Griffin and A.M. Griffin, "PCR technology: Current Innovations", CRC Press, Ann Arbor, (1994); and R.A. Eeles, and A.C. Stamps, "Polymerase Chain Reaction (PCR) the technique and its applications", R.G. Landes Company, Austin (1993).

The complementary strand portions of the hairpin polynucleotide are denatured by exposure to temperatures of 60°C to 105°C, preferably from about 90°C to 98°C.

Following the denaturation step, conditions are changed so that they are suitable for primer hybridization or annealing to the hairpin polynucleotide. This is normally performed by lowering the temperature such that the 3' portion of the primer binds to the primer.binding site on the hairpin polynucleotide. Depending on the sequence and length of the primer, the temperature for annealing can vary from approximately 20°C to 80°C, preferably from about 40°C to 72°C.

Following or concomitantly to the annealing step, conditions are established for primer extension. This is usually accomplished by adjusting the temperature to between about 60°C and 90°C, preferably about 68°C to about 76°C.

Primer extension is generally performed in a buffered aqueous solution, preferably at a pH of 7-9, most preferably about 8. Nucleoside triphosphates, e.g. dATP, dCTP, dGTP and TTP are also added to the synthesis mixture in adequate amounts and the resulting solution is heated to between about 60°C and 90°C, preferably about 68°C to about

76°C for about 1 to 10 minutes, preferably from 1 to 4 minutes, thereby resulting in primer extension. Conditions for primer extension are well known in the art and are applicable to the present invention. The extension reaction may in certain cases be enhanced by addition of a helicase, described above. Nucleoside triphosphate analogues, for example, 7-deaza-2'-deoxy-guanosine, inosine or nucleotides containing biotin or fluorescein can also be used.

Typically a three temperature cycle is used in the method of the present invention, for example denaturing at about 90-98°C, annealing at about 40-72°C and primer extension at about 68-76°C. However, other temperature cycles can also be used and may be advantageous for certain applications. For example, a five temperature cycle comprised of denaturation at about 90-98°C, annealing at about 40-72°C, extension at 68-76°C, denaturation at 90-98°C to separate primer extension product from hairpin polynucleotide, and extension at 68-76°C to complete any necessary intra strand copying may be used.

In one variation, the polymerase is thermally unstable DNA polymerase. In this instance it is necessary to isolate the DNA, including the target, product and copies thereof after each amplification cycle. Optionally, the DNA may be isolated after each cycle when using a thermally stable DNA polymerase.

In another variation, one or more nested primers are added to the amplification reaction mixture after one or more of the cycles. The probability of getting the correct amplicon is increased using nested primers. Because the first primer may hybridize to and be extended at unintended sites, use of additional primers (each closer to the hairpin end) after 10 to 20 cycles will selectively amplify the "correct" amplicon. Either low concentrations of the first primer are added relative to concentrations of second

or subsequent primers, or successive primers are chosen to hybridize at higher temperatures and the annealing temperature is increased upon addition of successive primers. Nested PCR is described in Pierre, et al, "Use of a reamplification protocol improves sensitivity of detection of *Mycobacterium tuberculosis* in clinical samples by amplification of DNA. *J. Clin. Micro.*, *29*:712-717, (1991).

A wide variety of methods can be used to provide the hairpin polynucleotide starting material. The hairpin amplification method of the present invention can be used to amplify hairpin polynucleotide obtained by these methods, some of which are described below. It is to be understood that hairpin amplification of target hairpins provided by other methods are also within the scope of the present invention. The "target" hairpin or polynucleotide is a hairpin or polynucleotide to be amplified. A "template" is the particular hairpin being used to amplify the target.

In a preferred embodiment, the target hairpin DNA is obtained by providing a sample containing a double stranded DNA and a "hairpin adapter" and ligating the hairpin adapter and double stranded DNA. A hairpin adapter has a phosphorylated 5' end and two regions which are sufficiently complementary so that they hybridize as shown in Figure 3. Preferably, the two regions are completely complementary. Under hybridizing conditions, the adapter forms the stem and loop structure characteristic of a hairpin. The hairpin adapter has three regions referred to as the cap or loop, stem and "end". Cap, loop and stem are defined in general as for a hairpin polynucleotide. The end is a region ligatable to a polynucleotide.

The end of the hairpin adapter stem can be blunt or can have an overhang. A "blunt end" of a double stranded DNA is an end in which the 5' terminal deoxynucleotide on

one strand is based paired with the 3' terminal deoxynucleotide on the other strand. A double stranded DNA with an overhang has a short single stranded tail extending from one end, i.e. one strand is longer than other at the one end as shown in Figure 4. Figure 4A shows a 5' 4-base overhang, in which the 5' end is four base pairs longer than the 3' end, and Figure 4B shows a 3' 2-base overhang, in which the 3' end is two base pairs longer than the 5' end. Blunt ends and overhangs may be produced by physically shearing DNA, but typically are produced by the action of restriction endonucleases on double stranded DNA. Examples of overhangs suitable for use in the present invention and the restriction endonucleases which generate them are given in the Table.

TABLE

Examples of Families of Adapters
Based on Compatible Overhangs

Examples of 5' 2-base overhangs

5'-CG	*Aci* I, *BsaH* I, *BspD* I, *BstB* I, *Cla* I *HinP1* I, *Hpa* II, *Mae* II, *Msp* I, *Nar* I *Psp1406* I, *Taq* I
5'-TA	*Ase* I, *Bfa* I, *Csp6* I, *Mse* I, *Nde* I

Examples of 5' 4-base overhangs

5'-AATT	*Apo* I, *EcoR* I, *Mun* I, *Tsp509* I
5'-AGCT	*Hind* III
5'-CATG	*Nco* I, *BspH* I
5'-CCGG	*Age* I, *BsaW* I, *BspE* I, *BsrF* I, *NgoM* I *SgrA* I, *Xma* I
5'-CGCG	*Asc* I, *Mlu* I, *BssH* II
5'-CTAG	*Avr* II, *Nhe* I, *Spe* I, *Xba* I
5'-GATC	*BamH* I, *Bcl* I, *Bgl* II, *Dpn* II, *Mbo* I *Sau3A* I, *Xho* II
5'-GCGC	*Kas* I
5'-GGCC	*Bsp120* I, *Cac82* I, *Eae* I, *Eag* I, *Not* I,
5'-GTAC	*Acc65* I, *BsiW* I, *BsrG* I
5'-TCGA	*Sal* I, *Xho* I, *PaeR7* I
5'-TGCA	*ApaL* I, *Ppu10* I
5'-TTAA	*Afl* II

Examples of 3' 2-base overhangs

AT-3'	*Pvu* I, *Pac* I
CG-3'	*Hha* I
GC-3'	*Sac* II

Examples of 3' 4-base overhangs

ACGT-3'	*Aat* II
AGCT-3'	*Sac* I
CATG-3'	*Nla* III, *Nsp* I, *Sph* I
GCGC-3'	*Bbe* I, *Hae* II
GGCC-3'	*Apa* I
GTAC-3'	*Kpn* I
TGCA-3'	*Nsi* I, *Pst* I, *Sse8387* I

Examples of blunt ends

Alu I, *BsaA* I, *BsaB* I, *BsrB* I, *Bst1107* I, *BstU* I
Dra I, *Ecl136* II, *Eco47* III, *EcoR V*, *Fsp* I
Hae III, *Hinc* II, *Hpa* I, *Msc* I, *Nae* I, *Nla IV*
Pme I, *Pml* I, *Pvu* II, *Rsa* I, *Sca* I, *Sma* I
SnaB I, *Srf* I, *Ssp* I, *Stu* I, *Swa* I, *Xmn* I

Example of a nonpalindromic sequence

BssS I C|TCGT G
G AGCA|C

Other families of restriction recognition sequences include palindromic penta-nucleotide sequences, interrupted palindromes and nonpalindromic sequences.

The end of an adapter is ligatable to a double stranded DNA with a blunt end if the adapter also has a blunt end. The end of an adapter with an overhang is ligatable to the end of a double stranded DNA with an overhang if they have compatible cohesive ends. (See examples in Table above). Ligitable overhangs are referred to as being cohesive.

Hairpin adapters may be of 8 to about 2000 thousand bases in length, but preferably are 30 to 60 bases in length. Suitable adapters may have unpaired single stranded loops containing from zero (adapters with less than 4 unpaired bases will still have a cap of 3-4 bases due to conformation constraints) to about 40 nucleotides, preferably from zero to about 20 bases. Suitable adapters have stems containing from about 3 to about 2000 base pairs, preferably from about 6 to about 30 base pairs.

Examples of suitable adapters include 5'-P-X-CAC GCT CTC CCT ATA GTG AGT CGT ATT AAG AGC GTG-Y-3' (SEQ ID NO: 12), 5'-P-X-CCC TAT AGT GAG TCG TAT TAA AAA ATT AAT ACG ACT CAC TAT AGG G-Y-3' (SEQ ID NO: 13), 5'-P-X-TCA GTC GTA GAT GCC AAC ATT TTT GTT GGC ATC TAC GAC TGA-Y-3' (SEQ ID NO: 14) and 5'P-X-GCA AGG TCT GAC AGT TTA TAA TAT TAT AAA CTG TCA GAC CTT GC-Y-3' (SEQ ID NO: 15). X and Y each independently represent from zero to about six bases selected from the group consisting of adenine, cytosine, guanine or thymidine and are selected so as to create a blunt end, a 3' overhang or a 5' overhang that can be ligated to an end, preferably a restricted end, of a single or double stranded polynucleotide to be amplified. In a specific example, X is AATT or CCGG and Y represents zero bases. The 5' end of all hairpin adapters are phosphorylated as indicated by P.

Adapters can be produced by any number of ways by one skilled in the art. For example, they can be chemically synthesized, produced by restriction of an appropriately constructed plasmid or produced as multiple fragments which are ligated together (Beyert *et al.*, "Nonhomologous DNA end joining of synthetic hairpin substrates in *Xenopus laevis* egg extracts. *Nucl. Acids Res.*, 22:1643-1650. (1994)).

In a preferred embodiment, a portion of the stem and/or loop of the hairpin adapter has a known sequence that is sufficiently long to hybridize with a primer which

can be used for primer extension by a template dependent polymerase. As described earlier, such sequences are typically from about 15-25, but can also be longer, for example up to about 200 nucleotides in length. The sequence of such a region is said to be "complementary to a sequencing primer." Suitable sequences for such regions include those complementary to standard primers such as the T7 promoter, the SP6 promoter, λgt10 forward, λgt10 reverse, λgt11 forward, λgt11 reverse, M13pUC forward, M13/pUC reverse, pBR322 clockwise and pBR322 counterclockwise. The ability to bind a primer to the hairpin adapter allows the determination of the sequence adjacent to the hairpin adapter following hairpin amplification. This is particularly useful in DNA, gene or chromosome walking into unknown flanking regions. Figure 5 shows the ligation of a hairpin adapter to restricted DNA (panels A & B) amplification of hairpin polynucleotide (panels C & D), and sequencing from the hairpin adapter primer site to obtain sequence information adjacent to the hairpin adapter (panels E & F). Primer binding sites are shown as double lines. Sequencing methods such as the Sanger method and cycle sequencing are known to those skilled in the art. Cycle sequencing is preferred.

The 5'-phosphorylated hairpin adapter may be ligated to single or double stranded polynucleotides using conditions well known to those skilled in the art. For example, T4 DNA ligase is commonly used, and protocols for blunt ligation and ligation of cohesive compatible end shave been described by S. Berger and A. R. Kimmel editors, "Guide to molecular cloning techniques"(Methods in Enzymology Vol 152, (1987)), and J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular cloning, a laboratory manual, second edition (Cold Spring Harbor Laboratory Press, (1989)). T4 RNA ligase may be used for joining single stranded RNA and DNA molecules as described in.P.

Romaniuk and C. Uhlenbeck, Methods in Enzymology Vol 100, pp 52-56. (1983)), and M. J. Moore and P. A. Sharp, Science, 256:992-997, (1992).

A "sample" can be biological material containing double stranded DNA that has a blunt end or a restricted end, single stranded DNA, or RNA. Biological material can be an isolate from prokaryotic or eukaryotic cells. Biological material can also be an isolate taken from a virus. Alternatively, a sample can simply be double stranded DNA having a blunt end or a restricted end, single stranded DNA or RNA. The polynucleotide can be genomic DNA from a eukaryote, prokaryote or virus, DNA from an organelle such as a mitochondria, or fragments and modifications thereof, or RNA from the same sources. Alternatively, the polynucleotide may be synthesized by chemical or enzymatic means.

Double stranded DNA for ligation to an adapter is typically obtained by digesting a large DNA fragment with one or more restriction endonucleases. Digestion by restriction enzymes leaves one or more double stranded DNA fragments, often thousands, having ends with known overhangs (or with ends known to be blunt). A hairpin adapter having an end ligatable to the one or more double stranded DNAs is provided and ligated to the double stranded DNA. Typically, more than one fragment having the same overhang (or blunt end) will result, and the adapter will ligate to each end of each of these fragment, resulting in a mixture of circular target DNAs for hairpin amplification.

Hairpin amplification methods are ideally suited to DNA, gene, or chromosome walking by amplifying unknown flanking regions. DNA is purified from the organism and divided into about 2 to 20 pools. Each pool is restricted with a different restriction endonuclease. Appropriate hairpin adapters are ligated to the DNA in each restricted

pool. A single primer (or a set of nested primers) is designed which hybridizes with a region of known sequence. The primer is used to perform a hairpin amplification reaction on an aliquot of each pool. The amplified products from each of the reactions is examined by separating on an agarose gel. In walking, the product producing the longest amplicon is preferred for sequencing. Using optimized PCR protocols, it may be possible to hairpin amplify segments of over 10 kilo bases. Sequencing using a primer to the hairpin adapter (all adapters are chosen to differ only at the ligatable end region) produces sequence information furthest away from the starting region of the amplification primer. Once the sequence adjacent to the adapter is determined, a new walking primer (or nested primers) is designed and synthesized and a second walk performed. The new primer is used to perform hairpin amplification of an aliquot of each pool except the one of the preceding walk. Walking may be repeated any number of times.

In another preferred embodiment, hairpin DNA is obtained from single stranded DNA by denaturation, followed by exposure to a mesophilic DNA polymerase such as *E. coli* DNA polymerase Klenow fragment at temperature from about 10°C to about 20°C, preferably at about 15°C for about 1-24 hours, preferably about 4 hours. Spontaneously formed 3' hybridization loops are extended as shown in Figure 6. This standard method for making the second strand of cDNA, is described in U. Gubler, "Second-strand cDNA synthesis: classical method", (Methods in Enzymology, 152:325-329, (1987).

In another preferred embodiment, the hairpin polynucleotide is obtained from a single stranded polynucleotide having a shepherd's crook at the 3' end. A "shepherd's crook" is a loop at one end of a single stranded polynucleotide which is a stable conformation at

ambient temperature and at normal ionic strengths, e.g. ionic strengths used for primer extensions. A 3'-shepherd's crook is a loop formed when at least the three, and preferably ten or more terminal nucleotides of a single stranded polynucleotide base pair back onto a portion of the single stranded polynucleotide as shown in Figure 7. Typically, the portion of the polynucleotide to which the terminal nuclectides bind is located near the 3'-end and forms a small loop, e.g. a loop of less than 100 bases, preferably 4 to 20 bases.

A 3'-shepherd's crook polynucleotide is transformed into a hairpin polynucleotide by exposure to a template dependent polymerase and nucleotide triphosphates under conditions for primer extension. Conditions for simple primer extension are well known in the art, (see S. L. Berger and A. R. Kimmel, "Guide to molecular cloning technique" (Methods in Enzymology, Vol 152, (1987) and J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular cloning, a laboratory manual, second edition (Cold Spring Harbor Laboratory Press, (1989)).

In another preferred embodiment the hairpin polynucleotide is obtained from a single stranded polynucleotide capable of forming a shepherd's crook at the 5' end (Figure 8). The definition of a 5'-shepherd's crook and loop size is the same as for 3'-shepherd's crook except that the loop is at the 5' end. The single stranded polynucleotide is combined with a template dependent polynucleotide polymerase, nucleoside triphosphates and an excess of a single polynucleotide primer capable of hybridizing to a site on the single stranded polynucleotide suitable for primer extension. The hybridizing site is preferably located as near the 3' end as possible. The combination is then exposed to conditions suitable for primer extension, as described above, thereby producing the hairpin polynucleotide. In certain applications it may be

necessary to add an additional step of denaturation to dissociate a polynucleotide capable of forming a 3' shepherd's crook which can be converted to the hairpin polynucleotide as described above.

In another preferred embodiment, the hairpin DNA is obtained from extension of a primer (probe) on a target polynucleotide as shown in Figure 17. In panel A is shown a single stranded target polynucleotide with regions A to D. At the 3' end of the probe is a sequence complementary to A on the target. At the 5' end of the probe is a sequence identical to that of B on the target. If the target polynucleotide is double stranded, it is denatured to yield single stranded target. The probe is then allowed to hybridize to the target, Figure 17 panel A. The probe is then exposed to a template dependent polynucleotide polymerase under conditions which allow probe extension at least to the 5' end of region D on the target, Figure 17 panel B. Denaturation leads to strand separation yielding the extension product in Figure C. This extension product is a 5' shepherd's crook and can be amplified using a primer D, complementary to extension product region D' as described above to yield the products shown in Figure 17 panel D.

In another preferred embodiment, the hairpin polynucleotide is obtained from mRNA (Figure 9). The mRNA is combined with a primer and reverse transcriptase or DNA polymerase under conditions suitable for reverse transcription, thereby producing cDNA hybridized to the mRNA (Figure 9B and C). Such conditions are well known in the art, and can be found in, for example S. L. Berger and A. R. Kimmel, "Guide to molecular cloning technique" (Methods in Enzymology, Vol 152, (1987) and J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular cloning, a laboratory manual, second edition (Cold Spring Harbor Laboratory Press, (1989)). The reaction product is exposed

to conditions suitable for removing the RNA from the cDNA, for example by combining with Ribonuclease H, or by treatment with alkali, as described in the above references (Figure 9C & D). The single stranded cDNA is combined with a mixture of hairpin adapters, (Figure 9E). Each adapter has a phosphorylated 5' end and a 3' overhang from 1 to about 15 nucleotides, preferably three or four nucleotides. The sequence of the overhang in the mixture contains all 64 three base combinations, or all 256 four base combinations of adenine, guanine, cytosine and thymidine (or uracil) (or analogues thereof, such as inosine, which may reduce the number of required adapters in a mixture). At least one adapter hybridizes to the cDNA under hybridizing conditions. This adapter can then be ligated to the cDNA, (Figure 9E), resulting in a single stranded DNA with a shepherd's crook the 3' end, (Figure 9F). This DNA can be converted into hairpin DNA by extension of the 3' end with DNA polymerase, (Figure 9G), as described above.

This method is applicable to a mixture of mRNAs, from which a mixture of cDNAs is produced. At least one adapter in the mixture can hybridize to each cDNA, allowing the production of a mixture of hairpin polynucleotides. This mixture of hairpin polynucleotides can be amplified using the primer used for first strand synthesis of the cDNA to amplify all cDNAs. If the hairpin adapter and the primer for cDNA synthesis are chosen or designed to possess appropriate restriction sites, then the amplified cDNAs may be easily cloned into a vector following restriction.

If amplification of a specific cDNA is desired, and a primer specific for that cDNA is known, then such a specific primer may be used for hairpin amplification. Following hairpin amplification and isolation of the amplified product, the 3' end of the cDNA may be sequenced using a primer complementary to a primer site on the

hairpin adapter. Direct sequencing without cloning is often desirable.

In another embodiment, the hairpin polynucleotide may be produced from any single stranded RNA or DNA. Hairpin amplification will require knowing the sequence of a region of the single stranded RNA or DNA for constructing a primer for hairpin amplification. This is shown in Figure 10. A hairpin adapter with a 5' overhang of about 6-18 bases, preferably 12, and a phosphorylated 5' end can be ligated to the 3' end of single stranded RNA or DNA using T4 RNA ligase as described (P. Romaniuk and O. Uhlenbeck, Methods in Enzymology, vol 100, (1983); and M. J. Moore, and P. A. Sharp, Science 256:992-997, (1992)). If single stranded DNA is used, the product is a 3'-shepherd's crook which is converted to a hairpin polynucleotide as described above. If single stranded RNA was used, a reverse transcriptase or DNA polymerase with reverse transcriptase activity such as that from *Thermus thermophilus* (described in *Amplifications* issue 12, Perkin Elmer Corporation (1994)) may be used for primer extension. Digestion with ribonuclease H or alkali would yield a 5' shepherd's crook which is converted to a hairpin polynucleotide as described above. Alternatively, a hairpin adapter with a 3' overhang of about 6-18 bases, preferably 12, can be ligated to the 5' end of a 5' phosphorylated single stranded RNA or DNA using T4 RNA ligase as described above. If single standed DNA is used, the product is a 5' shepherd's crook which is converted to a hairpin polynucleotide as described above. If single stranded RNA is used, a reverse transcriptase or appropriate DNA polymerase, as described above may be used for primer extension. Digestion with ribonuclease H or alkali would yield a 3' shepherd's crook, which is converted to a hairpin polynucleotide as described above.

In another preferred embodiment, the hairpin polynucleotide is obtained from enzymes that produce

hairpin DNA from double stranded DNA. For example, Vaccinia virus nicking-joining enzyme nicks, cleaves, and forms a hairpin loop at specific sites of suitable DNAs (M. K. Reddy and W. R. Bauer, "Activation of the Vaccinia virus nick-joining enzyme by trypsinization", J. Biol. Chem. 264:443-449, (1989)).

Another embodiment of the present invention is a method of detecting a double stranded polynucleotide in a sample suspected of containing a double stranded polynucleotide. The double stranded polynucleotide is preferably DNA, and can be restricted DNA, i.e. DNA obtained by digestion of DNA with one or more restriction endonucleases. A hairpin adapter having a phosphorylated 5' end suitable for ligating to the end of the double stranded polynucleotide, as described above, is combined with the sample under ligating conditions, as described above. The adapter ligates to the double stranded polynucleotide, if present, to produce a hairpin polynucleotide. The hairpin polynucleotide, if present, is then amplified by the hairpin amplification method of the present invention. A sufficient number of cycles are used so that amplified product can be detected if the starting polynucleotide is present. The presence or absence of amplified product is assessed, wherein its presence indicates that double stranded DNA is present in the sample. The presence of the amplified hairpin product can be detected by means known in the art, such as by using a radiolabeled primer, a primer with a fluorescent label or a biotinylated probe, according to methods known in the art.

Another embodiment is a method of detecting the presence or absence in a sample of at least one specific hairpin polynucleotide. The method comprises the same steps as the method of detecting a double stranded polynucleotide in a sample, except that the ligation step can be omitted.

Also provided is a kit comprising a ligase and a DNA hairpin adapter. Hairpin adapters are described above. The kit can additionally comprise a restriction endonuclease appropriate for the included hairpin adapter. In another aspect, the kit additionally comprises a restriction endonuclease and a template dependent polynucleotide polymerase. A sequencing primer complementary to a portion of the hairpin adapter can also be included. The kit can also comprise one or more of the following components: buffers, nucleotide triphosphates and control reagents. The invention is further illustrated by the following examples, which are not intended to be limiting in any way.

EXEMPLIFICATION

Adapters and primers shown in Figure 11 were synthesized commercially by automated DNA synthesis (Midland Certified Reagent Co., Midland, Texas). Adapters were phosphorylated on the 5'-end as indicated by a "P".

The target DNA for the exponential amplification reactions of the following examples is shown in Fig 12. 16S rDNA is shown as double line. The position of the restriction sites for *EcoR I* and *Age I* and the primer binding site for primer C72 are indicated.

Example 1

Hairpin Amplification of Target DNA Comprising Adapter C78 ligated to EcoR I Restricted *E. coli* DNA using Primer C72 and *Thermus aquaticus* DNA Polymerase, and Sequencing of Amplified product using Primer B96.

DNA Purification

DNA was purified from *E. coli* strain NM522. Cells were scraped from one plate and suspended in 1 ml of lysis buffer containing 10 mM Tris HCl, pH 7.6, 1.0 mM EDTA, 0.5% Tween 20 and 200 µg/ml proteinase K. The suspension was incubated at 55°C for 2 h. The proteinase K was inactivated by heating to 95°C for 10 minutes. Protein was removed from the lysis solution by phenol/chloroform extraction as described by Sambrook et al., 1989 (vol 3, pp E.3- E.4). DNA was precipitated by adding 1/10 vol sodium acetate (3 M pH 5.2) and 2 volumes ice cold absolute ethanol. The precipitate was collected by centrifugation. DNA was re-suspended in 10 mM Tris buffer pH 7.6.

Restriction - DNA from *E. coli* was restricted with EcoR I (NEB) using enzyme and buffers obtained from New England BioLabs. DNA was restricted overnight using 20 U of enzyme at 37°C. Completeness of the restriction reactions were checked by electrophoresis of a portion of the DNA on 1% agarose and staining with ethidium bromide. The digested DNA was extracted with phenol/chloroform and precipitated with ethanol (Sambrook et al., 1989). DNA was re-suspended in H_2O at a concentration of 0.5 µg/µl as determined by OD_{260}.

Ligation Reactions - The reaction mixture contained the following: 1.0 µg restricted *E. coli* DNA, 4 µM hairpin adapter C78, 1X Ligase Buffer, and 13 Weiss Units of T4 DNA

ligase in a final volume of 50 µl. The mixture was incubated at 16°C overnight. Completeness of the ligation reactions were checked by electrophoresis of 5 µl of the DNA on 4% agarose and staining with ethidium bromide.

Hairpin exponential amplification reaction. Figure 13A is a diagram showing the target DNA ligated to hairpin adapter C78. Hairpin adapter C78 is shown as a double line, the *E. coli* rDNA is shown as a single line. Location of primers C72 and B96 are shown by short single lines adjacent to their complementary target strands. The reaction mixture contained the following: 1 µM primer C72, approximately 250 ng of hairpin DNA, 2.5 units *Thermus aquaticus AmpliTaq®* DNA polymerase, 200 µM each of the dNTPs, 3 mM $MgCl_2$, and 1X buffer in a final volume of 82 µl. A wax bead hot start protocol was followed in which reagents other than polymerase and target DNA were added to the reaction tube. A wax bead was added and melted at 80°C for 5 minutes then solidified at 35°C for 10 minutes. The remaining reagents were added on top of wax. Reaction was initiated by heating to 95°C for 4 minutes followed by 40 cycles of denaturation 95°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 1.5 minutes with 5 seconds per cycle added to extension times. Reaction was followed by extension for 20 minutes at 72°C.

Sequence amplicon using primer B96. The hairpin adapter primer B96 was used to sequence from the hairpin adapter C78 in a 5' direction. Cycle sequencing was performed using a *fmol®* DNA Sequencing System (Promega Corp., Madison,WI) following manufacture's instructions. Figure 13B shows the adapter end of the expected amplicon. Hairpin adapter C78 is shown in bold uppercase. *E. coli* 16S rDNA fragment is shown in lower case. The full hairpin amplicon would extend to *E. coli* position 1509, for a total

length of about 857 base pairs. The sequence of the 4 bases of the hairpin cap is actually expected to contain the bases shown and the complementary sequence. Agarose gel electrophoresis of the amplicon showed a band at approximately 857 base pairs, but did not show a band at approximately 1714 base pairs. Based on this result, the product of amplification is a single stranded DNA in the form of a hairpin, and not a double stranded DNA typically observed in PCR amplifications.

Attempts to enhance reading off of adapter. The hairpin amplicon was digested with either S1 Nuclease or Exonuclease III so that the sequencing primer would have a single stranded target rather than a hairpin target. Hairpin amplicon produced as described above was digested with S1 Nuclease or Exonuclease III (Promega) using buffers and protocols supplied from the manufacturer. When sequencing from primer B96, over 300 bases could be read (bases 690-1010) for the unmodified amplicon and both the S1 nuclease or exonuclease III digested amplicons. The sequence read from the digested amplicons was clearer than the undigested amplicons.

Example 2

Hairpin Amplification of Target DNA Comprising Adapter C77 Ligated to EcoR I Restricted *E. coli* DNA using Primer C72 and *Thermus aquaticus* DNA Polymerase Stoffel Fragment, and Sequencing of Amplified product using Primer B36

DNA purification. DNA was purified from *E. coli* strain MC1061 as described in Example 1.

Restriction. DNA from *E. coli* was restricted with EcoR I (NEB) using enzyme and buffers obtained from New England

BioLabs. Five μg DNA were restricted overnight using 10 U of enzyme at 37°C. Completeness of the restriction reactions was checked by electrophoresis of a portion of the DNA on 1% agarose and staining with ethidium bromide. The digested DNA was extracted with phenol/chloroform and precipitated with ethanol (Sambrook et al., 1989). DNA was re-suspended in H_2O at a concentration of 0.5 μg/μl as determined by OD_{260}.

Ligation Reactions. The reaction mixture contained the following: 0.5 μg restricted *E. coli* DNA, 2 μM hairpin adapter C77, 1X Ligase Buffer, and 3 Weiss Units T4 DNA ligase in a final volume of 50 μl. The mixture was incubated at 15°C overnight. Completeness of the ligation reactions were checked by electrophoresis of 5 μl of the DNA on 4% agarose and staining with ethidium bromide.

Hairpin exponential amplification reaction. Because it was realized that the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase could potentially degrade template hairpin DNA, *Thermus aquaticus* DNA polymerase Stoffel fragment which lacks the 5'-3' exonuclease activity was used in the experiment of this example. Figure 14A is a diagram showing the target DNA ligated to hairpin adapter C77. Hairpin adapter C77 is shown as double lines, the *E. coli* DNA is shown as a single line. Location of primers C72 and B36 are shown by short single lines adjacent to their complementary target strands. The reaction mixture contained the following: 1 μM primer C72, 250 ng hairpin DNA, 2.5 units *Thermus aquaticus* DNA polymerase (*AmpliTaq*®) Stoffel fragment, 200 μM each of the dNTPs 3 mM $MgCl_2$ and 1X buffer in a final volume of 82 μl. A wax bead hot start protocol was followed in which reagents other than polymerase and target DNA were added to the reaction tube. A wax bead was added and melted at 80°C for 5 minutes then

solidified at 35°C for 10 minutes. The remaining reagents were added on top of wax. Reaction was initiated by heating to 95°C for 4 minutes, followed by 40 cycles of denaturation 95°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 1.5 minutes with 5 seconds per cycle added to extension times. Reaction was followed by extension for 20 minutes at 72°C.

Sequence of hairpin amplicon using primer B36. The hairpin amplicon was sequenced up to and around hairpin adapter C77 using primer B36. Cycle sequencing was performed using both a modified *Thermus aquaticus* DNA polymerase kit (fmol® DNA Sequencing System, Promega Corp., Madison,WI) and a modified *Thermococcus litoralis* DNA polymerase (CircumVent™ Thermal Cycle DNA Sequencing Kit, New England BioLabs, Beverly, MA) following manufacturers instructions. Figure 14B shows the adapter end of the expected amplicon. Hairpin adapter C77 is shown in bold uppercase. *E. coli* 16S rDNA fragment is shown in lower case. Full hairpin amplicon would extend to *E. coli* position 1509, for a total length of about 852 base pairs. The sequence of 21 bases in the hairpin cap is actually expected to contain bases shown and the complementary sequence. Primer B36 (*E. coli* 16S rRNA position 789-806, reverse orientation) gave the following read: matched *E. coli* sequence from 786-675. Sequence for hairpin adapter was GTGCGAGAATTATGCTGAnnnnnnnnnnnnnnnnnnCTTAA (SEQ ID NO: 16). The following sequence was readable past adapter GnTCCACATCGCCnCTTTACGCnTCTCTAG (SEQ ID NO: 17). Sequence beyond this point was indistinct. The sequencing gels were more distinct using the CircumVent™ Thermal Cycle DNA Sequencing Kit than the *fmol*® DNA Sequencing System.

Example 3

Hairpin Amplification of Target DNA Comprising Adapter C78 Ligated to EcoR I Restricted *E. coli* DNA using Primer C72 and *Thermus aquaticus* DNA Polymerase Stoffel Fragment, and Sequencing of Amplified product using Primer B36

DNA purification, restriction, and amplification were performed as described in Example 2. Ligation was also performed as described in Example 2, except that adapter C78 was used in place of adapter C77. Figure 15 is a diagram showing the target DNA ligated to hairpin adapter C78. Hairpin adapter C78 is shown as double lines and the *E. coli* DNA is shown as a single line. Location of primer C72 and B36 are shown by short single lines adjacent to their complementary target strands.

Sequence amplicon using primer B36. The amplicon was sequenced up to and around Hairpin adapter C78 using primer B36. Cycle sequencing was performed using both a fmol® DNA Sequencing System (Promega Corp., Madison,WI) and a CircumVent™ Thermal Cycle DNA Sequencing Kit (New England BioLabs, Beverly, MA) following manufacturers instructions.

The hairpin end of the expected amplified product is the same as in Example 1 (Figure 13B).

Sequencing using primer B36 (*E. coli* 16S rRNA position 789-806, reverse orientation) matched *E. coli* sequence from 786-675. The sequence for the adapter was GGGATATCACTCAGC nnAnTnnnnnnnnnnnnGCTGnGTGATATCCCTTAA (SEQ ID NO: 18). The following sequence was readable past adapter GGTCCACATCGCCACTTTACGCATCTCTAGACCTCCTTATGGCCACC (SEQ ID NO: 19).

Example 4

Hairpin Amplification of Target DNA Comprising Adapter D15 ligated to Age I restricted *E. coli* DNA using primer C72 and a *Thermococcus litoralis* DNA polymerase lacking exonuclease activity, and sequencing of amplified product using primers B36 and D13

DNA was purified from *E. coli* as described in Example 2.

Restriction. DNA from *E. coli* was restricted with Age I (NEB) using enzyme and buffers obtained from New England Biolabs. Five µg DNA were restricted overnight using 60 U of enzyme at 37°C. Completeness of the restriction reactions were checked by electrophoresis of a portion of the DNA on 1% agarose and staining with ethidium bromide. The digested DNA was extracted with phenol/chloroform and precipitated with ethanol (Sambrook et al., 1989). DNA was resuspended in H_2O at a concentration of 0.5 µg/µl as determined by OD_{260}.

Ligation Reactions. The reaction mixture contained the following: 0.5 µg restricted *E. coli* DNA, 4 µM hairpin adapter D15, 1X Ligase Buffer, and 1.2 Weiss U ligase in a final volume of 50 µl. The mixture was incubated at 16°C overnight and then at 65°C for 10 min. Completeness of the ligation reactions were checked by electrophoresis of 5 µl of the DNA on 4% agarose and staining with ethidium bromide.

Hairpin exponential amplification reaction. For this experiment, a different DNA polymerase was chosen to demonstrate that multiple polymerases can be used in the present invention. Because enzymes which produce strand displacement are thought to facilitate the amplification, an enzyme from Thermococcus *litoralis* was selected (H.M.

Kong, et al., *J. Biol. Chem.*, *268*:1965-1975 (1993)). The enzyme used lacked both 5'-3' and 3'-5' endonuclease activity. Figure 16A is a diagram showing the target DNA ligated to hairpin adapter D15. Hairpin adapter D15 is shown as a double line, and the *E. coli* rDNA is shown as a single line. Location of primers C72, B36 and D13 are shown by short single lines adjacent to their complementary target strands. The reaction mixture contained the following: 5 µl ligation reaction product, 0.2 mM dNTPs, 1 µM primer C72, 1X ThermoPol Reaction buffer [10 mM KCl, 20 mM tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100], and 2 Units Vent(exo-) DNA polymerase (New England BioLabs, Beverly, MA). A wax bead hot start protocol was followed in which reagents other than polymerase and target DNA were added to the reaction tube. A wax bead was added and melted at 80°C for 5 minutes then solidified at 35°C for 10 min. The remaining reagents were added on top of wax. Reaction was initiated by heating to 96°C for 4 minutes, followed by 40 cycles of denaturation 96°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes with 5 seconds per cycle added to extension times. Reaction was followed by extension for 20 minutes at 72°C.

Sequence amplicon using primer D13 and B36. The amplicon was sequenced up to and around Hairpin adapter D15 using primer B36. The hairpin adapter primer D13 was used to sequence from the hairpin adapter D15 in a 5' direction. Cycle sequencing was performed using a CircumVent™ Thermal Cycle DNA Sequencing Kit (New England BioLabs, Beverly, MA) following manufacturers instructions.

Figure 16B shows the adapter end of the expected amplicon. Hairpin adapter D15 is shown in bold uppercase. *E. coli* 16S rDNA fragment is shown in lower case. The full hairpin would extend to *E. coli* position 1509, for a total

length of about 810 base pairs. The sequence of the 4 bases in the hairpin cap is expected to contain the bases shown and the complementary sequence.

Sequencing using primer B36 (*E. coli* 16S rRNA position 789-806, reverse orientation) matched *E. coli* sequence from 782-718. The sequence for the loop was AGTCAGCATCTACGGTTGTTTTnnnnnnnnnnAGATGCTG (SEQ ID NO: 20). The sequence beyond this point was indistinct.

Sequencing using primer D13 (identical to 3'-18 bases of D15 and therefore complementary to 5'-bases 5-22 of adapter) gave clear sequence from *E. coli* positions 740 to 1085 (about 350 bases), except that base 770 was indistinct (N) and GGs at 902-3 and 941-2 were compressed.

Some experiments attempting hairpin amplification of hairpin DNA using the adapters described above, or additional adapters, failed when positive controls did not work. For example, when self ligation of an adapter could not be demonstrated (as seen by doubling of molecular weight of self ligated vs unligated adapter on 4% agarose gel), or when the polymerase control did not produce a product (PCR reaction was performed using same polymerase, target DNA, buffer, and same primer as used for hairpin amplification, but with addition of a second primer chosen to amplify a majority of total hairpin DNA), then no hairpin amplification was seen. Other experiments failed in which there were no positive controls. It is believed that these failures were methodological and due to lack of adapter ligation, or improper reagents for amplification reaction where the PCR control would also have failed.

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed is:

1. A method of producing at least one copy of a hairpin polynucleotide comprising the steps of:

a) providing at one least hairpin polynucleotide;

b) combining the hairpin polynucleotide of (a) with a template dependent polynucleotide polymerase, nucleoside triphosphates and an excess of a single polynucleotide primer that is capable of hybridizing to a site on the 3' portion of the hairpin polynucleotide and that is suitable for primer extension such that an extension product synthesized from the single primer can serve as a template for synthesis of an extension product of the single primer;

c) disrupting the base-pairing of the hairpin polynucleotide, thereby disassociating the two complementary segments of the hairpin polynucleotide;

d) exposing the polynucleotide of (c) to conditions allowing at least the 3' portion of the polynucleotide primer to hybridize to the primer site on the hairpin polynucleotide; and

e) exposing the polynucleotide hybridized to the primer to conditions suitable for primer extension, thereby producing a complementary copy of the hairpin polynucleotide as the extension product.

2. The method of Claim 1 wherein steps (c), (d) and (e) are repeated at least once, thereby producing an amplification of the hairpin polynucleotide and the extension product.

3. The method of Claim 2 wherein the hairpin polynucleotide is hairpin DNA and the template dependent polynucleotide polymerase is a DNA polymerase.

4. The method of Claim 3 wherein a nested primer is added after a step (c), (d) or (e).

5. The method of Claim 3 wherein the hairpin DNA is obtained by the following steps:

a) providing a sample comprising a double stranded DNA;

b) providing a DNA hairpin adapter with a phosphorylated 5' end and which is ligatable to an end of the double stranded DNA;

c) ligating the double stranded DNA and the adapter DNA, thereby producing the hairpin DNA.

6. The method of Claim 5 wherein the adapter has a blunt end or an end ligitable to restricted DNA.

7. The method of Claim 5 wherein the adapter is a 5' phosphorylated oligonucleotide having a sequence selected from the group consisting of:
X-CACGCTCTCCCTATAGTGAGTCGTATTAAGAGCGTG-Y,
X-CCCTATAGTGAGTCGTATTAAAAAATTAATACGACTCACTATAGGG-Y,
X-TCAGTCGTAGATGCCAACATTTTTGTTGGCATCTACGACTGA-Y and
X-GCAAGGTCTGACAGTTTATAATATTATAAACTGTCAGACCTTGC-Y,
wherein X and Y each independently represent from zero to six bases selected from the group consisting of adenine, cytosine, guanine and thymine.

8. The method of Claim 7 wherein X is AATT or CCGG and Y represents zero nucleotides.

9. The method of Claim 5 wherein the double stranded DNA is obtained from double stranded DNA which has been cleaved with one or more restriction enzymes.

10. The method of Claim 9 wherein the sample comprises a mixture of DNAs.

11. The method of Claim 10 wherein the adapter additionally comprises DNA having a sequence that is complementary to a sequencing primer.

12. The method of Claim 11 wherein at least one amplified hairpin DNA is separated from the mixture.

13. The method of Claim 11 wherein the DNA is sequenced using a primer complementary to a primer binding site on the hairpin adapter.

14. The method of Claim 3 wherein the hairpin DNA is obtained from a single stranded polynucleotide by the steps of:

a) exposing a single stranded polynucleotide to denaturing conditions;

b) exposing the product of (a) to self-hybridizing conditions; and

c) exposing the product of (b) to conditions suitable for extending the 3' end of the polynucleotide, thereby producing the hairpin polynucleotide.

15. The method of Claim 2 wherein the hairpin polynucleotide is obtained by the following steps:

a) providing a single stranded polynucleotide capable of forming a 3' shepherd's crook;

b) exposing the single stranded polynucleotide to conditions suitable for forming a shepherd's crook; and

c) exposing the product of (b) to conditions suitable for extending the 3' end of the polynucleotide, thereby producing the hairpin polynucleotide.

16. The method of Claim 15 wherein the single stranded polynucleotide is DNA.

17. The method of Claim 16 wherein the single stranded polynucleotide capable of forming a 3' shepherd's crook is obtained by the steps of:

a) providing single stranded DNA and a DNA hairpin adapter with a phosphorylated 5' end and a 5' overhang; and

b) ligating the 3' end of the single stranded DNA and the 5' end of the hairpin adapter, thereby producing a single stranded polynucleotide capable of forming a 3' shepherd's crook.

18. The method of Claim 16 wherein the single stranded DNA capable of forming a 3' shepherd's crook is obtained by the steps of:

a) providing single stranded 5' phosphorylated RNA and a DNA hairpin adapter with a 3' overhang;

b) ligating the 3' end of the adapter to the 5' end of the RNA;

c) binding to the single stranded portion of the product a primer suitable for product extension of b); and

d) exposing to conditions suitable for extension of the primer with a polymerase suitable for primer

24. The method of Claim 3 wherein the hairpin DNA is obtained by the following steps:

a) combining target RNA with reverse transcriptase under conditions appropriate to produce cDNA from the target mRNA;

b) subjecting the product of (a) to reagents and conditions suitable for removing the target mRNA from the cDNA;

c) combining the cDNA of (b) with a mixture of hairpin DNAs, referred to as hairpin adapters, having:

i) a phosphorylated 5' end; and

ii) a 3' overhang having the nucleotide sequence XYZ, wherein X, Y, and Z are each independently selected from the group consisting of adenine, guanine, cytosine and thymidine;

d) subjecting the combination of (c) to hybridizing conditions, thereby hybridizing the 5' end of at least one hairpin adapter to the 3' end of the cDNA;

e) ligating the cDNA to the hairpin adapter thereby forming a single stranded DNA having a hairpin loop; and

f) exposing the product of step (e) to conditions suitable for extending the 3' end, thereby forming the hairpin DNA.

25. The method of Claim 24, wherein the primer hybridizes to more than one cDNA.

26. The method of Claim 24, wherein the primer hybridizes to a specific cDNA.

27. The method of Claim 14 wherein the target RNA is at least one RNA in a mixture of RNAs.

28. The method of Claim 3 wherein the hairpin DNA is provided by combining the double stranded DNA with an enzyme which adds a loop onto an end of the double stranded DNA under conditions suitable for adding the loop.

29. The method of Claim 28 wherein the enzyme is Vaccinia virus nicking joining enzyme.

30. A method of detecting a hairpin polynucleotide in a sample comprising the steps of:

a) providing a sample suspected of containing a hairpin polynucleotide;

b) combining the sample of (a) with a template dependent polynucleotide polymerase, nucleoside triphosphates and an excess of a single polynucleotide primer that is capable of hybridizing to a site on the hairpin polynucleotide and that, if present, is suitable for primer extension such that an extension product synthesized from the single primer can serve as the template for the synthesis of an extension product of the single primer;

c) disrupting the base-pairing of the hairpin polynucleotide, thereby dissociating the two complementary portions of the hairpin polynucleotide, if present; and

d) exposing the polynucleotide of (c), if present, to conditions allowing at least the 3' portion of the polynucleotide primer to hybridize to the primer site on the hairpin polynucleotide;

e) exposing the product polynuclectide hybridized to the primer to conditions suitable for primer extension, thereby producing a complementary copy of the hairpin polynucleotide, if present;

f) repeating steps (c), (d) and (e) a sufficient number of times to amplify the hairpin polynuclectide and the extension product, if present; and

g) detecting the presence of amplified product, if present, wherein the presence of amplified product indicates the presence of the hairpin polynucleotide in the sample.

31. The method of Claim 30 wherein the hairpin polynuclectide is hairpin DNA and the template dependent polynucleotide polymerase is a DNA polymerase.

32. A method of detecting double stranded DNA in a sample comprising the steps of:

a) providing a sample suspected of containing a double stranded DNA;

b) providing hairpin DNA, referred to as an adapter, with a phosphorylated 5' end and which is ligatable to an end of the double stranded DNA;

c) ligating the double stranded DNA, if present, and the adapter DNA, thereby producing a hairpin DNA; and

d) detecting the hairpin DNA, if present, by the method of Claim 19, wherein the presence of amplified hairpin DNA indicates that the double stranded DNA is present in the sample.

33. The method of Claim 32 wherein the double stranded DNA in the sample is restricted.

34. A method of producing at least one copy of a hairpin polynucleotide comprising the steps of:

a) providing at least one hairpin polynucleotide;

b) combining the hairpin polynucleotide of (a) with a template dependent polynucleotide polymerase, nucleoside triphosphates and an excess of a single polynucleotide primer that is capable of hybridizing to a site on the 3'portion of the hairpin polynucleotide and that is suitable for primer extension such that a hairpin extension product synthesized from the single primer can serve as the template for the synthesis of a hairpin extension product of the single primer;

c) disrupting the base-pairing of the hairpin polynucleotide, thereby dissociating the two halves complementary segments of the hairpin polynucleotide;

d) exposing the polynucleotide of (c) to conditions allowing at least the 3' portion of the polynucleotide primer to hybridize to a primer site on the hairpin polynucleotide; and

e) exposing the polynucleotide hybridized to the primer to conditions suitable for primer extension, thereby producing a hairpin complementary copy of the hairpin polynucleotide as the extension product.

35. The method of Claim 34 wherein step (c) is repeated at least once, thereby producing an amplification of the hairpin polynucleotide and the hairpin extension product.

36. A method of producing at least one copy of a hairpin polynucleotide comprising the steps of:

a) providing at one least hairpin polynucleotide;

b) combining the hairpin polynucleotide of (a) with a template dependent polynucleotide polymerase, nucleoside triphosphates and an excess of a single polynucleotide primer that is capable of hybridizing to a site on the hairpin polynucleotide suitable for primer extension into the duplex portion of the stem and such that an extension product synthesized from the single primer can serve as the template for the synthesis of an extension product of the single primer;

c) disrupting the base-pairing of the hairpin polynucleotide, thereby dissociating the two complementary segments of the hairpin polynucleotide;

d) exposing the polynucleotide of (c) to conditions allowing at least the 3' portion of the polynucleotide primer to hybridize to a primer site on the hairpin polynucleotide; and

e) exposing the polynucleotide hybridized to the primer to conditions suitable for primer extension, thereby producing a complementary copy of the hairpin polynucleotide as the extension product.

37. The method of Claim 36 wherein steps (c) through (e) are repeated at least once, thereby producing an amplification of the hairpin polynucleotide and the extension product.

38. A method of producing at least one copy of a hairpin polynucleotide comprising the steps of:

a) providing at one least hairpin polynucleotide;

b) dissociating the complementary segments of the hairpin polynucleotide; and

c) forming in the presence of nucleoside triphosphates and a template dependent polynucleotide polymerase a hairpin extension product of a polynucleotide primer which has been hybridized to a site on the 3' portion of the target hairpin polynucleotide suitable for copying into the duplex portion of the hairpin stem, an hairpin extension product having a sufficient number of bases such that a region complementary to the polynucleotide primer is formed.

39. The method of Claim 38 wherein steps (b) through (c) are repeated at least once, thereby producing an amplification of the hairpin polynucleotide and the hairpin extension product.

40. A kit comprising:

a) a hairpin DNA, referred to as an adapter, with a phosphorylated 5' end and which is ligatable to a blunt end or a restricted end of a double stranded target DNA; and

b) a ligase.

41. The kit of Claim 40 wherein the adapter is a 5' phosphorylated oligonucleotide selected from the group consisting of:

X-CACGCTCTCCCTATAGTGAGTCGTATTAAGAGCGTG-Y,

X-CCCTATAGTGAGTCGTATTAAAAAATTAATACGACTCACTATAGGG-Y,

X-TCAGTCGTAGATGCCAACATTTTTGTTGGCATCTACGACTGA-Y and

X-GCAAGGTCTGACAGTTTATAATATTATAAACTGTCAGACCTTGC-Y,

wherein X and Y each independently represent from zero to six bases selected from the group consisting of adenine, cytosine, guanine and thymine.

42. The kit of Claim 41 wherein X is AATT or CCGG and y represents zero nucleotides.

43. The kit of Claim 40 wherein the adapter further comprises a sequencing primer complementary to a site on the hairpin adapter.

44. The kit of Claim 43 further comprising a restriction endonuclease.

45. The kit of Claim 44 further comprising a template dependent polynucleotide polymerase.

FICATION OF SUBJECT MATTER
G12Q1/68 C12P19/34 C07H21/04

International Patent Classification (IPC) or to both national classification and IPC

SEARCHED

n searched (classification system followed by classification symbols)

on searched other than minimum documentation to the extent that such documents are included in the fields searched

ta base consulted during the international search (name of data base and, where practical, search terms used)

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DE,A,44 38 630 (PACHMANN) 2 May 1996 see the whole document ---	1-3,30, 31,34-39
WO,A,93 17127 (THE STATE OF OREGON) 2 September 1993 see the whole document ---	1-46
WO,A,94 20639 (THE UNIVERSITY OF MEDICINE DENTISTRY OF NEW JERSEY) 15 September see page 47, paragraph 4 - page 48, line 1; figure 5E ---	1-3,30, 31,34-39 5-33, 40-45
WO,A,93 09246 (UNIVERSITY OF IOWA RESEARCH FOUNDATION) 13 May 1993 see claims 17,18; figures FIG.2-2 ---	1-46
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documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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defining the general state of the art which is not d to be of particular relevance

ument but published on or after the international e

which may throw doubts on priority claim(s) or ited to establish the publication date of another r other special reason (as specified)

referring to an oral disclosure, use, exhibition or ins

published prior to the international filing date but the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

ual completion of the international search	Date of mailing of the international search report
October 1996	31. 10. 96
of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Osborne, H

(second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	EP,A,0 292 802 (HOECHST AG.) 30 November 1988 see the whole document	14-17, 19,20, 23-29
A	EP,A,0 469 755 (SYNTEX (USA) INC.) 5 February 1992 see figure W	1-46
A	WO,A,94 03624 (AUERBACH) 17 February 1994	
A	WO,A,93 12245 (IGEN INC.) 24 June 1993	

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		ZA-A-	9209319	24-05-93

Fig. 1A

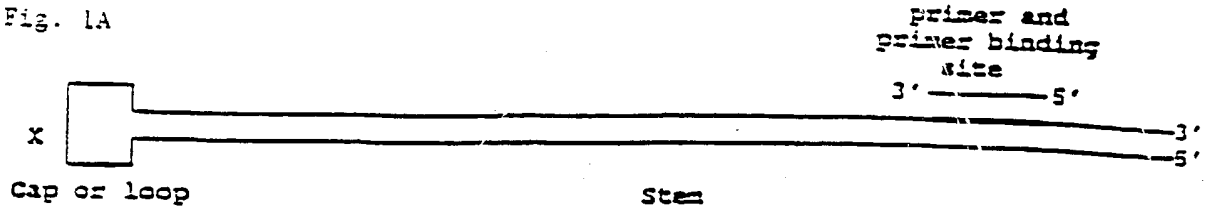


Fig. 1B

Fig. 1C

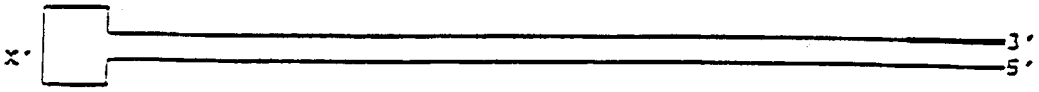


Fig. 1D

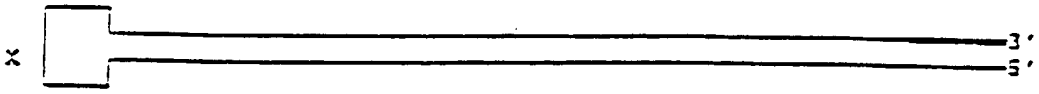


Fig. 1E

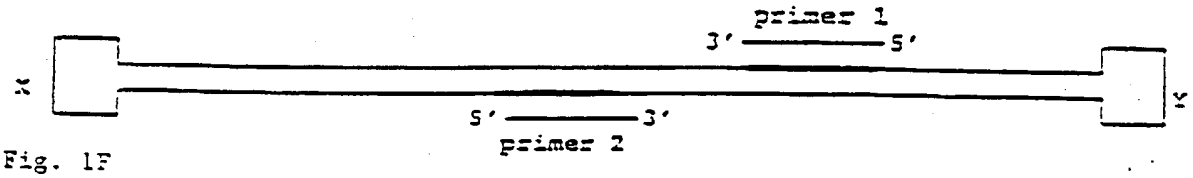


Fig. 1F

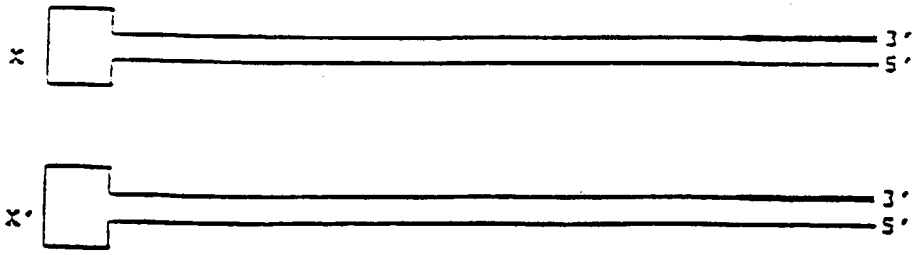


Fig. 1G

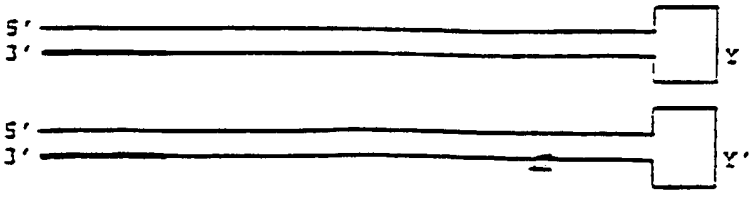
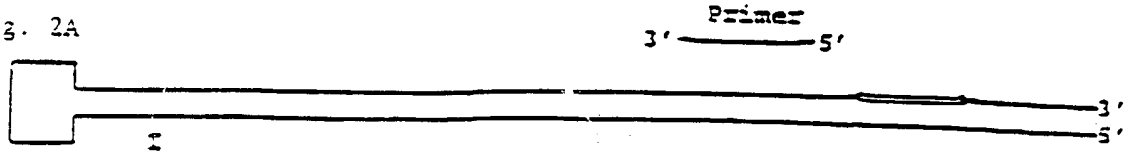
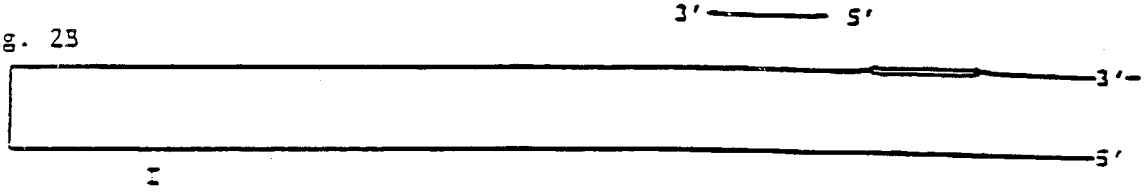


Fig. 2A



Disassociate

Fig. 2B



Hybridize

Fig. 2C

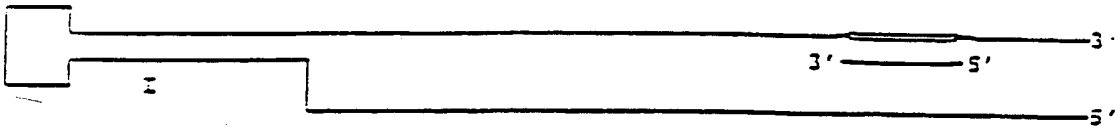
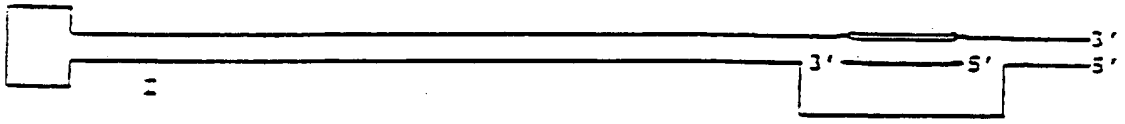


Fig. 2D



Primer extension

Fig. 2E

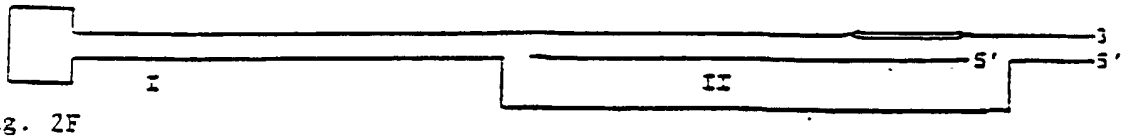


Fig. 2F

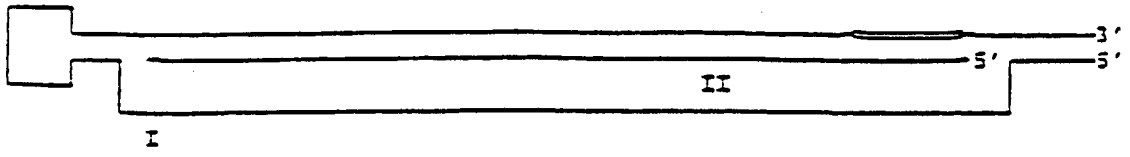
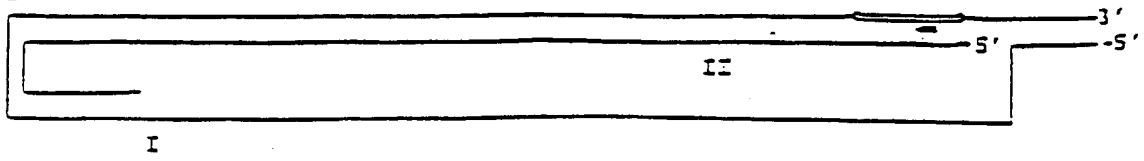


Fig. 2G



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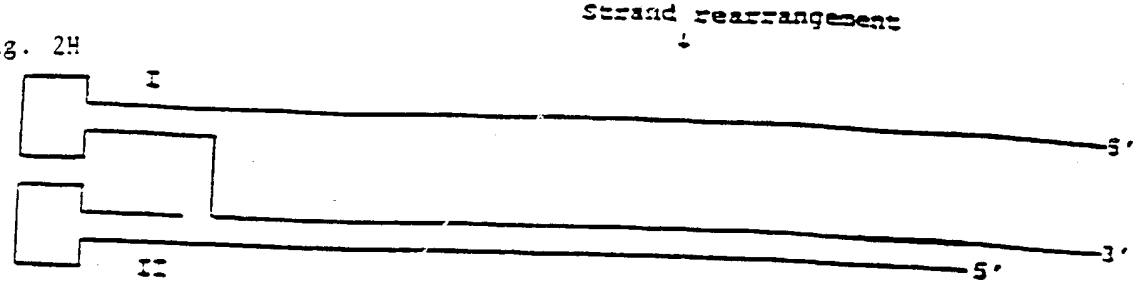


Fig. 2I

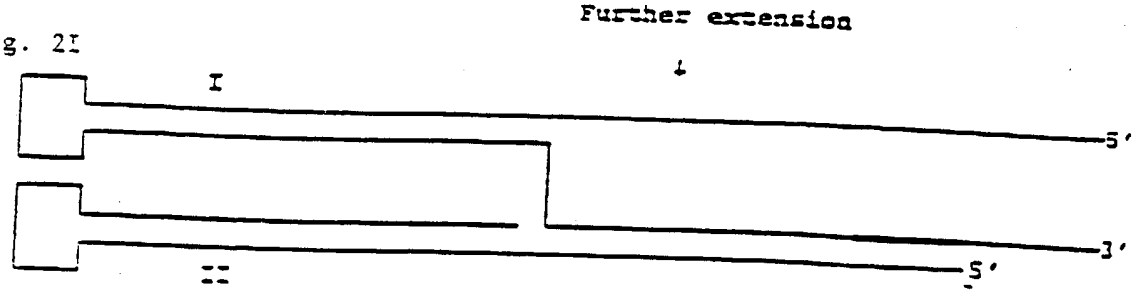


Fig. 2J

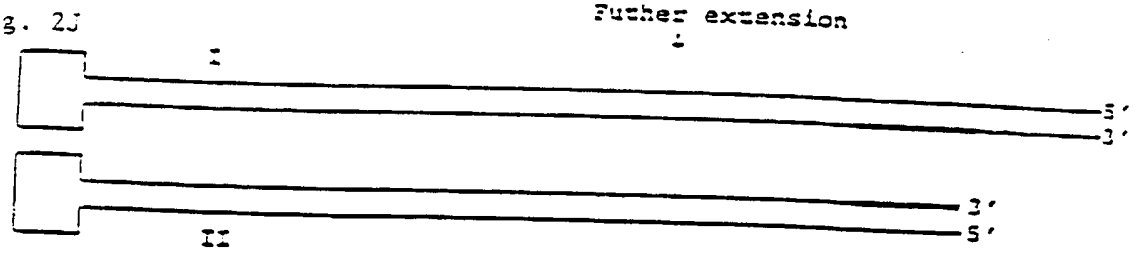


Fig. 2K

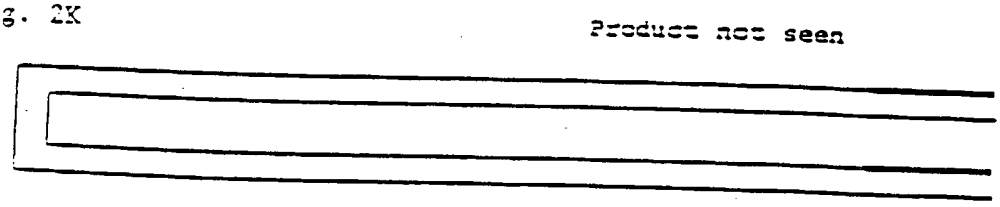
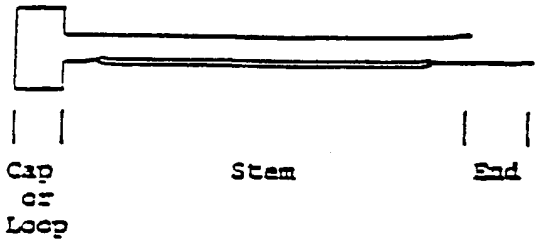


Fig. 3



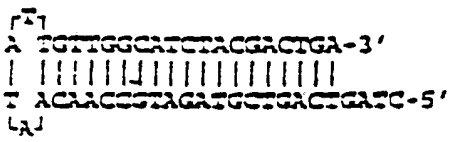


Fig. 4A

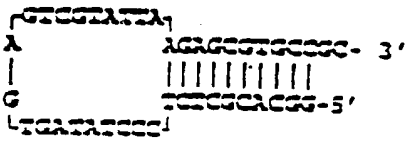
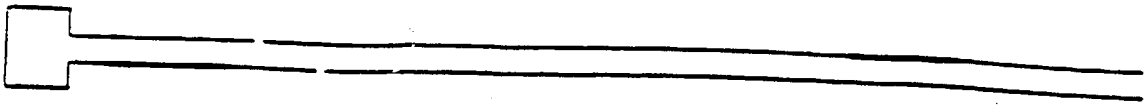


Fig. 4B

Fig. 5A



Ligate

Fig. 5B

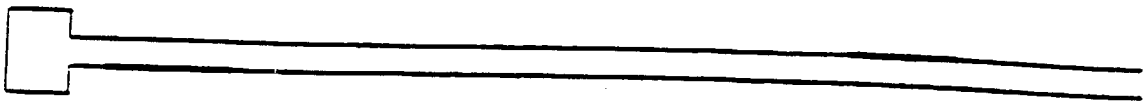
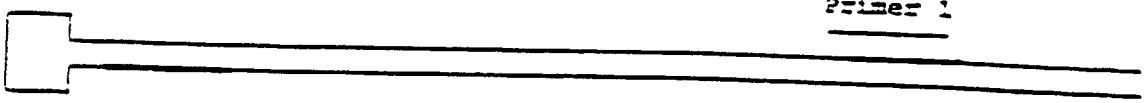


Fig. 5C



Amplify

Fig. 5D

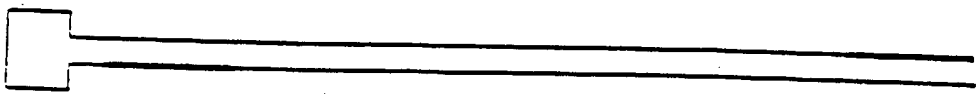
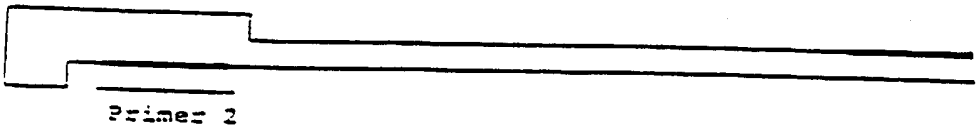


Fig. 5E



Sequence

Fig. 5F

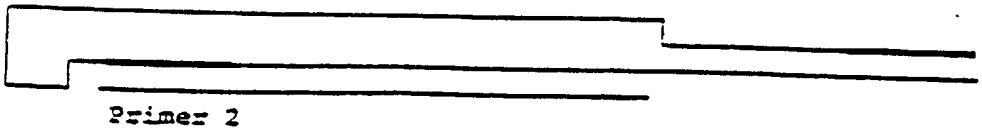


Fig. 6A

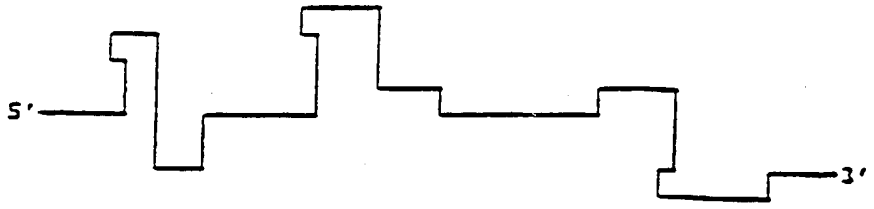


Fig. 6B

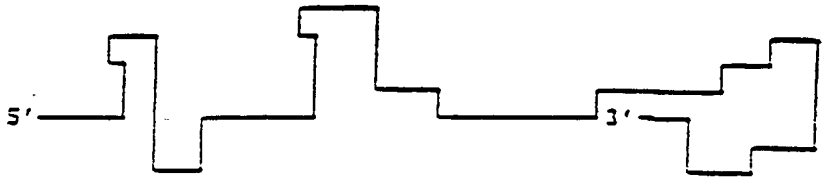


Fig. 6C

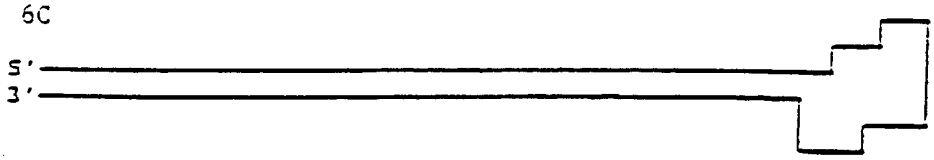


Fig. 7A

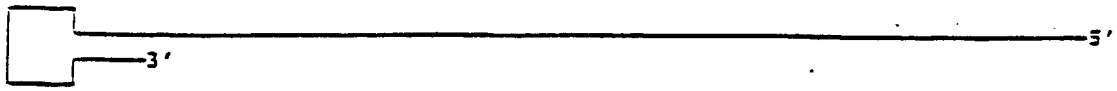


Fig. 7B

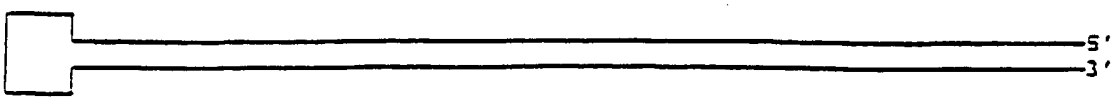


Fig. 8A

Fig. 8B

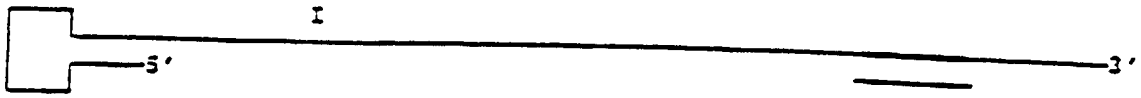


Fig. 8C

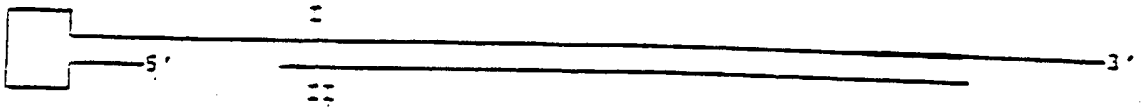


Fig. 8D

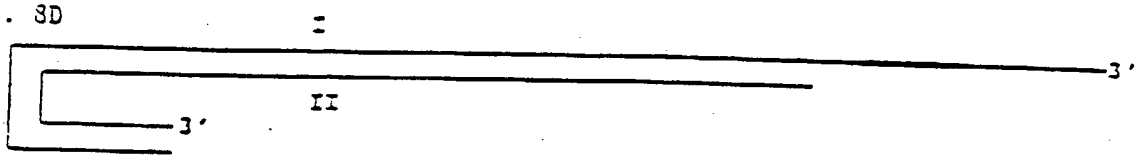


Fig. 8E

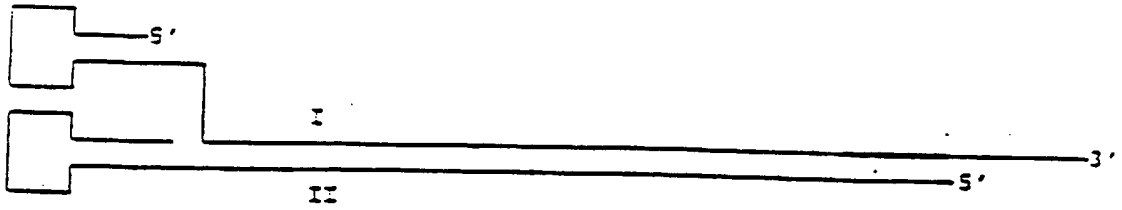


Fig. 8F

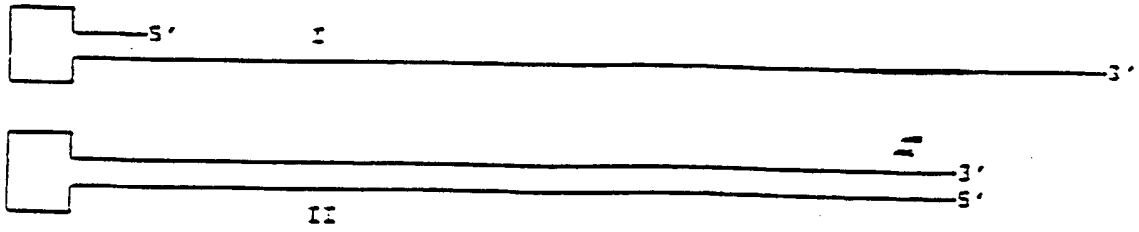


Fig. 9A

Fig. 9B

Fig. 9C

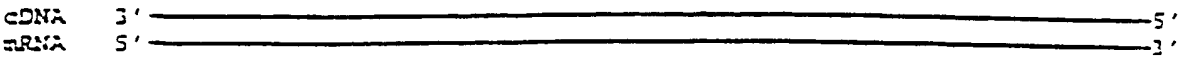


Fig. 9D

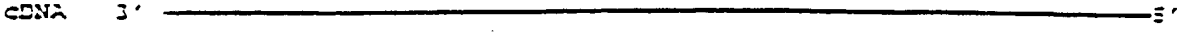


Fig. 9E

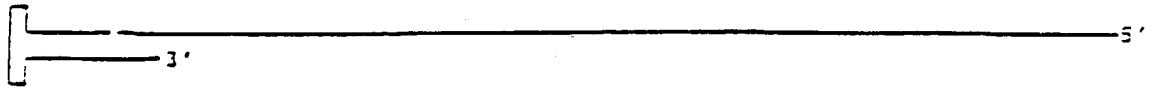


Fig. 9F

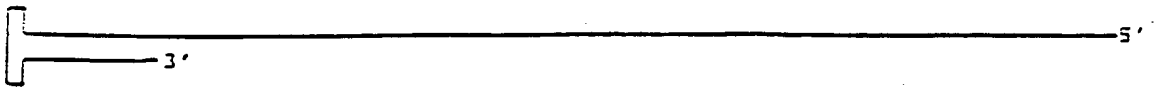


Fig. 9G

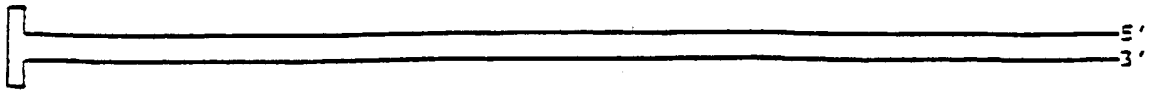


Fig. 10A

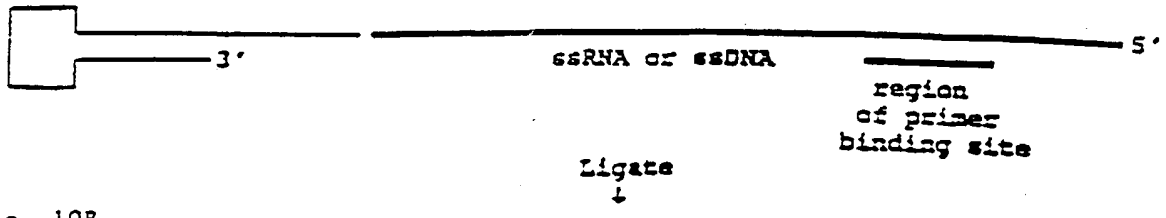


Fig. 10B

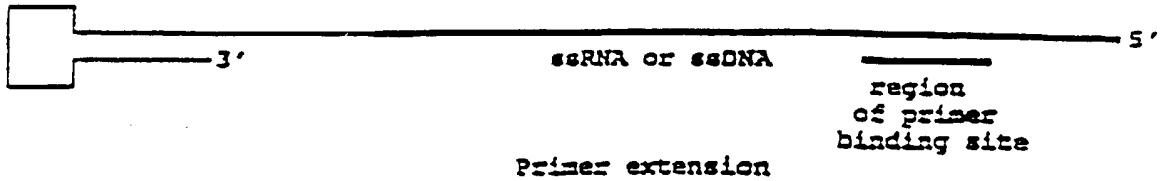


Fig. 10C

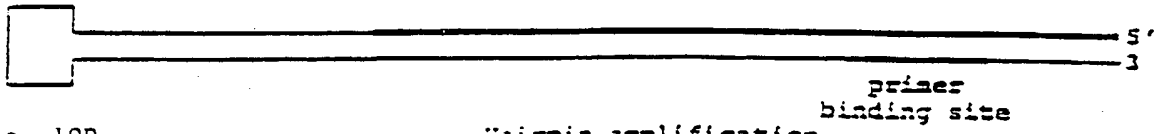


Fig. 10D

Hairpin amplification

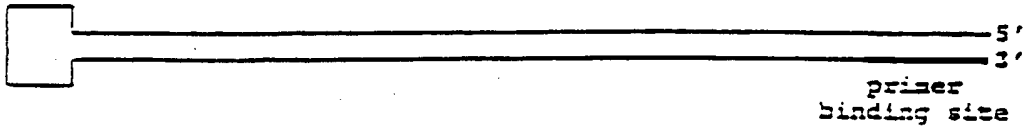


Fig. 10E

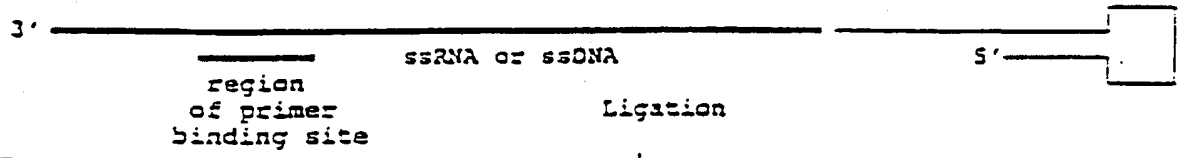


Fig. 10F

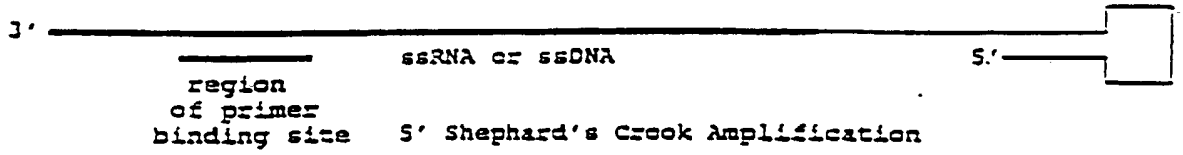


Fig. 10G

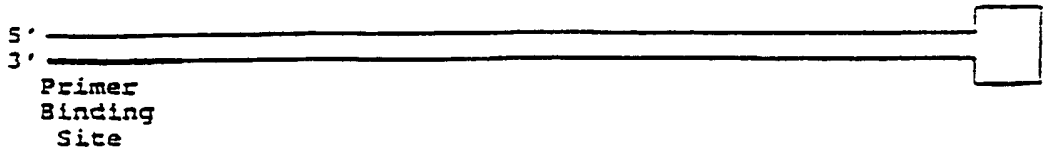


Fig. 11

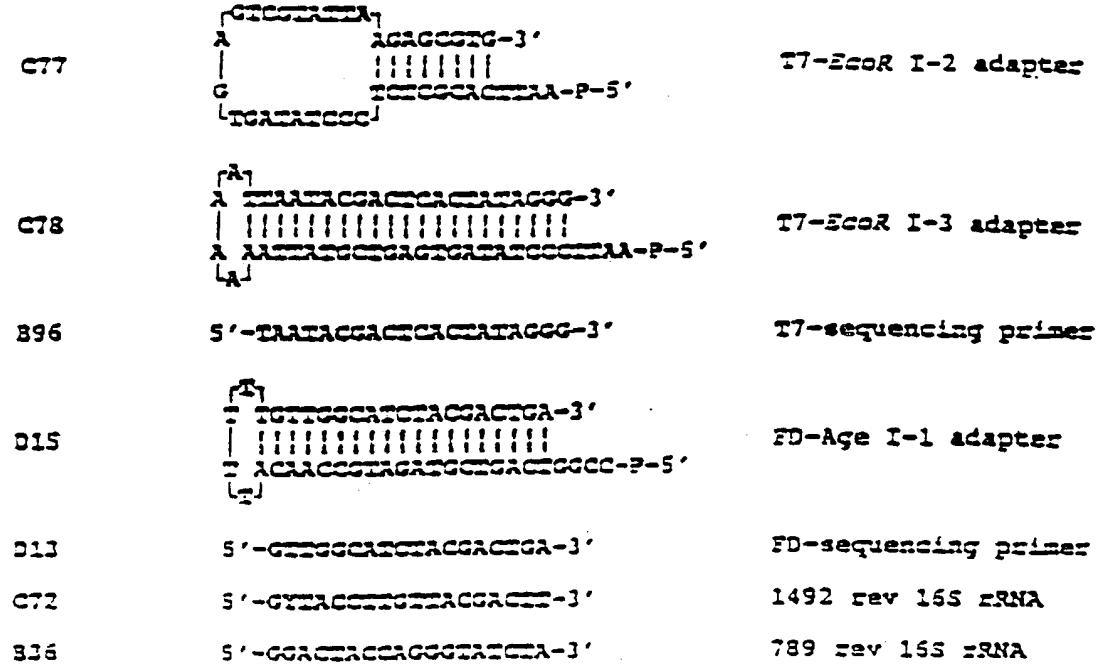


Fig. 12

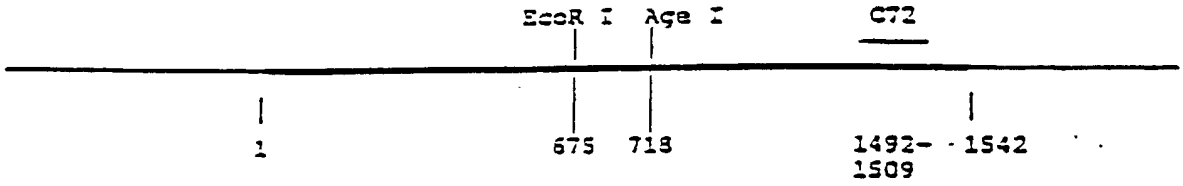


Fig. 13A

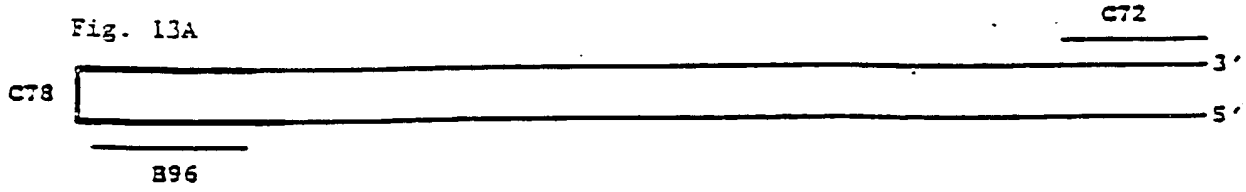


Fig. 13B

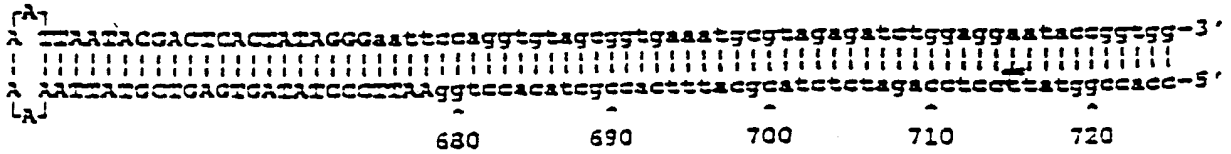


Fig. 14A

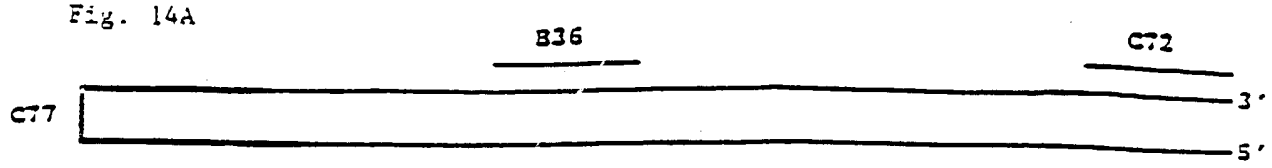


Fig. 14B

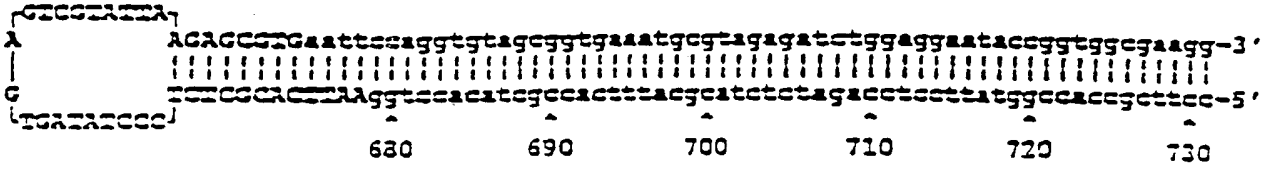


Fig. 15

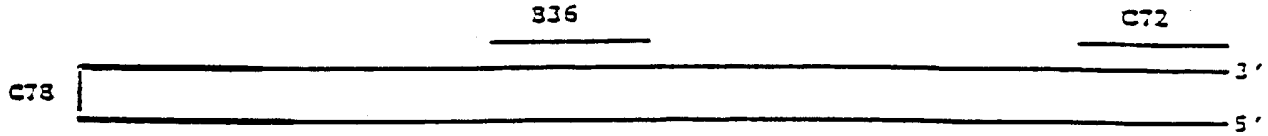


Fig. 16A

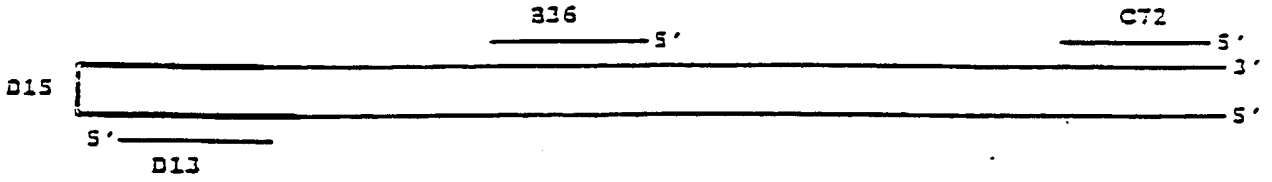


Fig. 16B

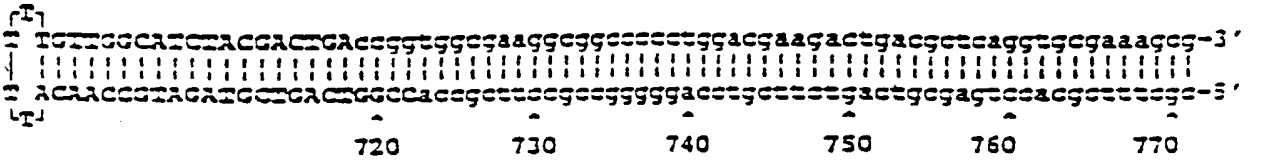


Fig. 17A

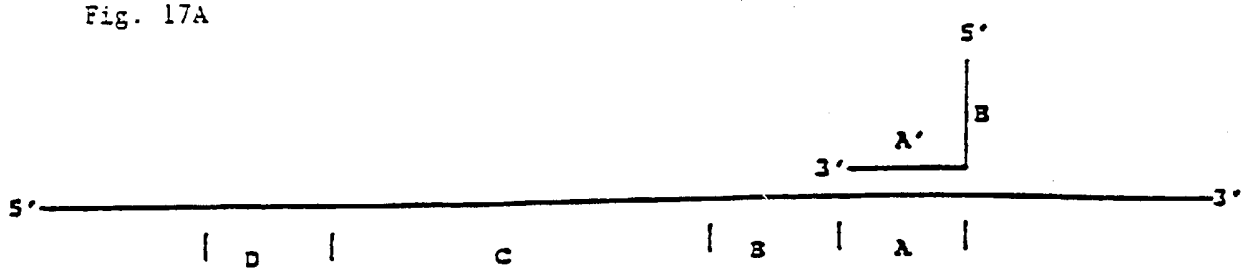


Fig. 17B

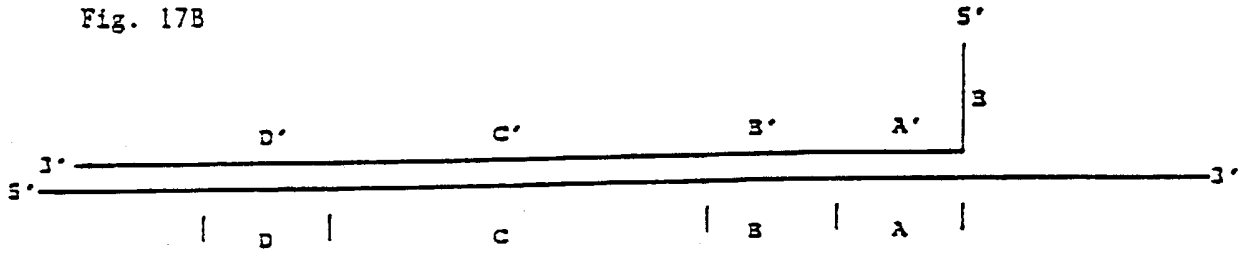


Fig. 17C

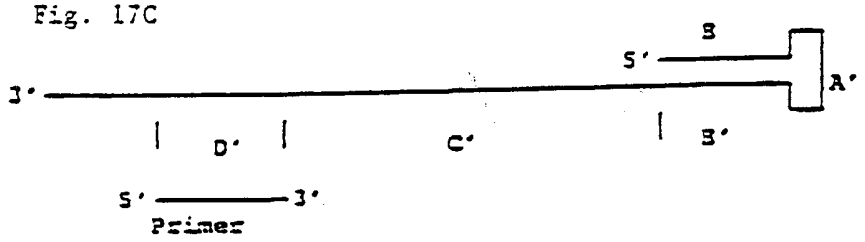


Fig. 17D